02-08CIP(213.1152UCIP)

POLYMERIC OLIGONUCLEOTIDE PRODRUGS

Cross-Reference to Related Applications

This application claims the benefit of priority from U.S. Provisional Patent Application Serial No. 60/462,070, filed April 13, 2003, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present inventions relates to polymeric oligonucleotide prodrugs useful as therapeutic agents. Compositions and methods of using such prodrugs are also provided.

BACKGROUND

It is well known that most of the bodily states in multicellular organisms, including most disease states, are effected by proteins. Such proteins, either acting directly or through their enzymatic or other functions, contribute in major proportion to many diseases and regulatory functions in animals and man. For disease states, classical therapeutics have generally focused upon interactions with such proteins in efforts to moderate their disease-causing or disease potentiating functions. In newer therapeutic approaches, modulation of the actual production of such proteins is desired. By interfering with the production of proteins, the maximum therapeutic effect may be obtained with minimal side effects. It is therefore a general object of such therapeutic approaches to interfere with or otherwise modulate gene expression, which would lead to undesired protein formation.

One method for inhibiting specific gene expression is with the use of oligonucleotides, especially oligonucleotides that are complementary to a specific target messenger RNA (mRNA) sequence. Generally, nucleic acid sequences complementary to the products of gene transcription (*e.g.*, mRNA) are designated "antisense", and nucleic acid sequences having the same sequence as the transcript

or being produced as the transcript are designated "sense". See, *e.g.*, Crooke, 1992, *Annu. Rev. Pharmacol. Toxicol.*, 32: 329-376. An antisense oligonucleotide can be selected to hybridize to all or part of a gene, in such a way as to modulate expression of the gene. Transcription factors interact with double-stranded DNA during regulation of transcription. Oligonucleotides can serve as competitive inhibitors of transcription factors to modulate their action. Several recent reports describe such interactions (see Bielinska, A., *et al.*, 1990, *Science*, 250: 997-1000; and Wu, H., *et al.*, 1990, *Gene* 89: 203-209).

Molecular strategies are being developed to down-regulate unwanted gene expression. Recently, the use of modified oligonucleotide compounds has developed into a promising method of treatment against such diseases as viral infections, inflammatory and genetic disorder and significantly, cancer. Antisense DNAs were first conceived as alkylating complementary oligodeoxynucleotides directed against naturally occurring nucleic acids (Belikova, *et al.*, *Tetrahedron Lett.* 37:3557-3562, 1967). Zamecnik and Stephenson were the first to propose the use of synthetic antisense oligonucleotides for therapeutic purposes. (Zamecnik & Stephenson, 1978, *Proc. Natl. Acad. Sci. U.S.A.*, 75:285-289; Zamecnik & Stephenson, 1978, *Proc. Natl. Acad. Sci. U.S.A.*, 75:280-284). They reported that the use of an oligonucleotide 13-mer complementary to the RNA of Rous sarcoma virus inhibited the growth of the virus in cell culture. Since then, numerous other studies have been published manifesting the *in vitro* efficacy of antisense oligonucleotide inhibition of viral growth, *e.g.*, vesicular stomatitis viruses (Leonetti *et al.*, 1988, *Gene*, 72:323), herpes simplex viruses (Smith *et al.*, 1987, *Proc. Natl. Acad. Sci. U.S.A.* 83:2787), and influenza virus (Seroa; *et al.*, 1987, *Nucleic Acids Res.* 15:9909).

Oligonucleotides have also found use in among others, diagnostic tests, research reagents *e.g.* primers in PCR technology and other laboratory procedures. Oligonucleotides can be custom synthesized to contain properties that are tailored to fit a desired use. Thus numerous chemical modifications have been introduced into oligomeric compounds to increase their usefulness in diagnostics, as research reagents and as therapeutic entities.

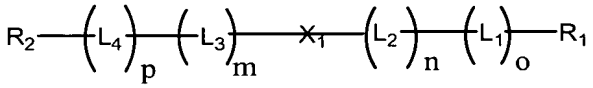
Although oligonucleotides, especially antisense oligonucleotides show promise as therapeutic agents, they are very susceptible to nucleases and can be rapidly degraded before and after they enter the target cells making unmodified antisense oligonucleotides unsuitable for use in *in vivo* systems. Because the enzymes responsible for the degradation are found in most tissues, modifications to the oligonucleotides have been made in an attempt to stabilize the compounds and remedy this problem. The most widely tested modifications have been made to the back-bone portion of the oligonucleotide compounds. See generally Uhlmann and Peymann, 1990, *Chemical Reviews* 90, at pages 545-561 and references cited therein. Among the many different back bones made, only phosphorothioate showed significant antisense activity. See for example, Padmapriya and Agrawal, 1993, *Bioorg. & Med. Chem. Lett.* 3, 761. While the introduction of sulfur atoms to the back bone slows the enzyme degradation rate, it also increases toxicity at the same time. Another disadvantage of adding sulfur atoms is that it changes the back bone from achiral to chiral and results in 2^n diastereomers. This may cause further side effects. Still more disadvantages of present antisense oligonucleotides are that they may carry a negative charge on the phosphate group which inhibits its ability to pass through the mainly lipophilic cell membrane. The longer the compound remains outside the cell, the more degraded it becomes resulting in less active compound arriving at the target. A further disadvantage of present antisense compounds is that oligonucleotides tend to form secondary and high-order solution structures. Once these structures are formed, they become targets of various enzymes, proteins, RNA, and DNA for binding. This results in nonspecific side effects and reduced amounts of active compound binding to mRNA. Other attempts to improve oligonucleotide therapy have included adding a linking moiety and polyethylene glycol. See for example, Kawaguchi, *et al.*, Stability, Specific Binding Activity, and Plasma Concentration in Mice of an Oligodeoxynucleotide Modified at 5'-Terminal with Poly(ethylene glycol), *Biol. Pharm. Bull.*, 18(3) 474-476 (1995), and US Patent No. 4,904,582. In both of these examples, the modifications involve the use of linking moieties that are permanent in nature in

an effort to stabilize the oligonucleotide against degradation and increase cell permeability. However, both of these efforts fail to provide any efficacy.

Due to the inadequacies of the present methods, there exists a need to improve stability and resistance to nuclease degradation as well as decrease toxicity and increase binding affinity to mRNA of oligonucleotide compounds. The current oligonucleotide therapy is expensive. This is mainly due to the degradation problem. Thus, there is a real need to protect the antisense oligonucleotide compounds against degradation, prevent the formation of high-order structures and at the same time deliver sufficient amounts of active antisense oligonucleotide compounds to the target. This invention provides such improvements.

SUMMARY OF THE INVENTION

In one aspect of the invention there are provided oligonucleotide prodrugs of formula (I):



(I)

wherein:

R_1 and R_2 are independently H or a polymer residue;

L_1 and L_4 are independently selected releasable linking moieties;

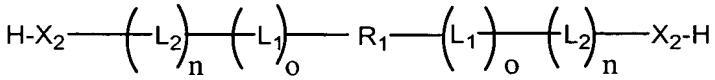
L_2 and L_3 are independently selected spacing groups;

X_1 is a nucleotide residue or an oligonucleotide residue;

m, n, o and p are independently zero or a positive integer, provided that either (o + n) or (p + m) $\geq$ 2.

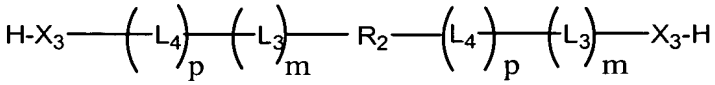
Another aspect of the invention includes bifunctional compounds that are formed when R_1 and/or R_2 are polymer residues which include both an alpha and an omega terminal linking group as described herein so that two oligonucleotides are linked to the polymeric delivery systems provided. Examples of this embodiment include oligonucleotides joined to the polymer systems through their respective 3'-, 5'- terminal groups, *e.g.* 3'-bis oligonucleotide conjugates or 5'-bis oligonucleotide conjugates, or a conjugate formed by linking a first oligonucleotide

via the 3'- terminal to the 5'-terminal of a second oligonucleotide. Examples of such polymer conjugates are illustrated below as formulas (i), (ii), (iii) and (iv):



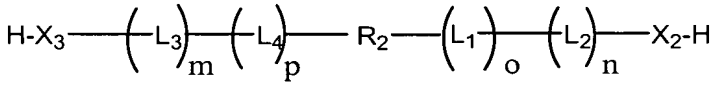
(i)

bis-3'-oligonucleotide,



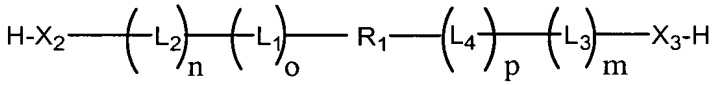
(ii)

bis-5'-oligonucleotide,



(iii)

bis-5', 3'-oligonucleotide, and



(iv)

bis-3', 5'-oligonucleotide

wherein all variables are as described above.

For purposes of the present invention, the term "residue" shall be understood to mean that portion of a biologically active compound *i.e.* an oligonucleotide, more specifically an antisense oligonucleotide, which remains after it has undergone a substitution reaction in which the prodrug carrier has been attached.

For purposes of the present invention, the term "polymeric residue" or "PEG residue" shall each be understood to mean that portion of the polymer or PEG which remains after it has undergone a reaction with a modified oligonucleotide compound.

For purposes of the present invention, the term "alkyl" shall be understood to include straight, branched, substituted, *e.g.* halo-, alkoxy-, nitro-, C_{1-12} alkyls, C_{3-8} cycloalkyls or substituted cycloalkyls, etc.

For purposes of the present invention, the term "substituted" shall be understood to include adding or replacing one or more atoms contained within a functional group or compound with one or more different atoms.

For purposes of the present invention, substituted alkyls include carboxyalkyls, aminoalkyls, dialkylaminos, hydroxyalkyls and mercaptoalkyls; substituted alkenyls include carboxyalkenyls, aminoalkenyls, dialkenylaminos, hydroxyalkenyls and mercaptoalkenyls; substituted alkynyls include carboxyalkynyls, aminoalkynyls, dialkynylaminos, hydroxyalkynyls and mercaptoalkynyls; substituted cycloalkyls include moieties such as 4-chlorocyclohexyl; aryls include moieties such as napthyl; substituted aryls include moieties such as 3-bromo-phenyl; aralkyls include moieties such as toluyl; heteroalkyls include moieties such as ethylthiophene; substituted heteroalkyls include moieties such as 3-methoxy-thiophene; alkoxy includes moieties such as methoxy; and phenoxy includes moieties such as 3-nitrophenoxy. Halo- shall be understood to include fluoro, chloro, iodo and bromo.

The term "sufficient amounts" or "effective amounts" for purposes of the present invention shall mean an amount which achieves a therapeutic effect as such effect is understood by those of ordinary skill in the art.

Some of the chief advantages of the present invention include novel polymeric oligonucleotide prodrugs that demonstrate increased stability and resistance to nuclease degradation, increased solubility, increased cell permeability and decreased toxicity.

Another advantage of the compounds of the invention is that a variety of polymeric prodrug platforms are releasably attached to the modified oligonucleotide compounds. This advantage allows for the artisan to design a drug conjugate that can be manipulated to include various moieties between the polymeric residue and the attached oligonucleotide that can effect the rate of hydrolysis of the prodrug. The artisan thus has the ability to include substituents that allow for modulation of the rate of hydrolysis of the prodrug.

Methods of making and using the compounds, such as in methods of treating cancers or malignancies, and conjugates described herein are also provided. It is

also contemplated that inventive polymeric oligonucleotide prodrugs be administered together with (simultaneously and/or sequentially) any other suitable anticancer agent.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 schematically illustrates methods of preparing the PEGylated oligonucleotides of compounds **3** and **5**.

FIG. 2 schematically illustrates methods of preparing the PEGylated oligonucleotides of compounds **7** and **9**.

FIG. 3 schematically illustrates methods of preparing the PEGylated oligonucleotides of compounds **11**, **12** (SEQ ID NO: 1) and **14** (SEQ ID NO: 1).

FIG. 4 schematically illustrates a method of preparing the PEGylated oligonucleotide of compound **16** (SEQ ID NO: 1).

FIG. 5 schematically illustrates methods of preparing the PEGylated oligonucleotides of compounds **17** (SEQ ID NO: 2), **18** (SEQ ID NO: 3) and **19** (SEQ ID NO: 4), from AS1 (SEQ ID NO:2), AS2 (SEQ ID NO:3) and AS3 (SEQ ID NO:4).

FIG. 6 schematically illustrates methods of preparing the PEGylated oligonucleotides of compounds **21** (SEQ ID NO: 1) and **22** (SEQ ID NO: 2).

FIG. 7 schematically illustrates methods of preparing the PEGylated oligonucleotides of compounds **24** (SEQ ID NO: 1) and **26** (SEQ ID NO: 1).

FIG. 8 schematically illustrates methods of preparing the PEGylated oligonucleotides of compounds **28** (SEQ ID NO: 1), **29** (SEQ ID NO: 2), **30** (SEQ ID NO: 3) and **31** (SEQ ID NO: 4), from AS1 (SEQ ID NO:2), AS2 (SEQ ID NO:3) and AS3 (SEQ ID NO:4).

FIG. 9 schematically illustrates methods of preparing the PEGylated oligonucleotides of compounds **33** (SEQ ID NO: 1) and **35** (SEQ ID NO: 1).

FIG. 10 illustrates the inhibitory effects of compound **14** and compound **28** on PC3 cell growth. 0.4 x 10^4 cells were seeded in 96-well plates, treated either with complexes of compound **14** or compound **28** (400 nM) and Lipofectin (15 μg/ml) for 24 hrs in Opti-MEM and then in complete media without complexes. Cellular

viability was determined daily, and absorbances were measured at 570 nm. Data are presented as the average ± standard deviation; n=4. Curves are as follows:
Control is marked by ♦ and a dotted curve;
Compound 28 at 400nM is marked by ▬ and a solid curve;
Compound 14 at 400nM is marked by ▲ and a dashed curve;
Compound 28 at 200nM is marked by ▬ and a dashed curve;
Compound 14 at 200nM is marked by ■ and a dotted curve.

FIG. 11A provides a summary of ROS production (from flow cytometric analysis) by compound **14** and compound **28** oligonucleotides, by detecting the oxidation of cell-permeable 2',7'-dihydrodichlorofluorescein diacetate to fluorescent 2',7'-dichlorofluorescein (**DCF**). PC3 cells were treated with oligonucleotides (400 nM)/Lipofectin (15μg/ml) complexes for 24 hrs, and assayed after 3 days, as described. Fold increases in mean fluorescence channel were normalized against untreated cells. Experiments were done in triplicate and data are presented as mean ± standard deviation (n = 3).

FIG. 11B provides a summary of ROS production (from flow cytometric analysis) by compound **14** and compound **28** oligonucleotides, by detecting the oxidation of hydroethidium (**HE**) to ethidium (**E**), which subsequently intercalates into DNA with fluorescence detectable by flow cytometry. PC3 cells were treated with oligonucleotides (400 nM)/Lipofectin (15μg/ml) complexes for 24 hrs, and assayed after 3 days, as described. Fold increases in mean fluorescence channel were normalized against untreated cells. Experiments were done in triplicate and data are presented as mean ± standard deviation (n = 3).

FIG. 12 is a Western Blot results confirming inhibition of bcl-2 protein expression by compound **14** in the presence of Lipofectin. PC3 cells were treated with compound **14** oligonucleotide (200, 400 and 800 nM) in the presence (+Lipo) and absence (-Lipo) of Lipofectin for 24 hrs in Opti-MEM, and then for a further 67 hrs in complete media. Protein samples (30-40 μg of protein/lane) were analyzed by Western blotting as described in the Material and Methods, with tubulin used as a control protein species. "C" marks the control.

DETAILED DESCRIPTION OF THE INVENTION

Accordingly, the present invention provides for polymer-linked oligonucleotide prodrugs useful having many practical uses, including uses as diagnostic and analytic reagents, as research and investigational tools, both *in vitro* and *in vivo*, and as therapeutic agents. In order to more fully appreciate the scope of the present invention, the following terms are defined. The artisan will appreciate that the terms, "nucleic acid" or "nucleotide" apply to deoxyribonucleic acid ("DNA"), ribonucleic acid, ("RNA) whether single-stranded or double-stranded, unless otherwise specified, and any chemical modifications thereof. An "oligonucleotide" is generally a relatively short polynucleotide, *e.g.*, ranging in size from about 2 to about 200 nucleotides, or more preferably from about 10 to about 30 nucleotides in length. The oligonucleotides according to the invention are generally synthetic nucleic acids, and are single stranded, unless otherwise specified. The terms, "polynucleotide" and "polynucleic acid" may also be used synonymously herein.

Modifications to the oligonucleotides of the invention optionally include, for example, the addition to or substitution of selected nucleotides with functional groups or moieties that permit covalent linkage of an oligonucleotide to a desirable polymer, and/or the addition or substitution of functional moieties that incorporate additional charge, polarizability, hydrogen bonding, electrostatic interaction, and functionality to an oligonucleotide. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodouracil, backbone modifications, methylations, base-pairing combinations such as the isobases isocytidine and isoguanidine, and analogous combinations. Oligonucleotide modifications can also include 3' and 5' modifications such as capping.

The term "antisense," as used herein, refers to nucleotide sequences which are complementary to a specific DNA or RNA sequence that encodes a gene product or that encodes a control sequence. The term "antisense strand" is used in reference to a nucleic acid strand that is complementary to the "sense" strand. In the normal

operation of cellular metabolism, the sense strand of a DNA molecule is the strand that encodes polypeptides and/or other gene products. The sense strand serves as a template for synthesis of an messenger RNA ("mRNA") transcript (an antisense strand) which, in turn, directs synthesis of any encoded gene product. Antisense nucleic acid molecules may be produced by any art-known methods, including synthesis by ligating the gene(s) of interest in a reverse orientation to a viral promoter which permits the synthesis of a complementary strand. Once introduced into a cell, this transcribed strand combines with natural sequences produced by the cell to form duplexes. These duplexes then block either the further transcription or translation. In this manner, mutant phenotypes may be generated. The designations "negative" or (-) are also art-known to refer to the antisense strand, and "positive" or (+) are also art-known to refer to the sense strand

For example, if it is intended to downregulate expression of an mRNA transcript in a cell or cells, the antisense oligonucleotide is introduced into a cell. Once introduced into a cell, the antisense oligonucleotide hybridizes to the corresponding mRNA sequence through Watson-Crick binding, forming a heteroduplex. Once the duplex is formed, translation of the protein coded by the sequence of bound mRNA is inhibited. Thus, antisense oligonucleotides are also employed in the art as probes, *e.g.*, hybridization probes, generally linked to a tag or label, as well as being used to provide precise downregulation of the expression of specific cellular products or genetic regulatory elements for both investigational and therapeutic purposes.

For purposes of the present invention, the use of the singular or plural is not meant to be limiting of the numerical number of the referenced item or object. Thus, the use of the singular to refer to a cell, polymer or drug does not imply that only one cell is treated, only one molecule is prepared or employed, and/or only one drug is employed, and the use of the plural does not exclude application to a single referenced item, unless expressly stated.

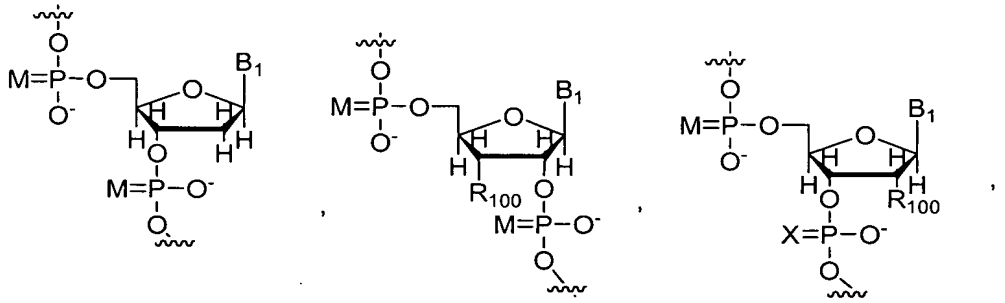
For purposes of the present invention, the term "residue" shall be understood to mean that portion of a biologically active compound, such as an oligonucleotide, which remains after it has undergone a reaction in which the prodrug carrier portion has been attached by modification of *e.g.*, an available hydroxyl or amino group, to form, for example, an ester or amide group, respectively.

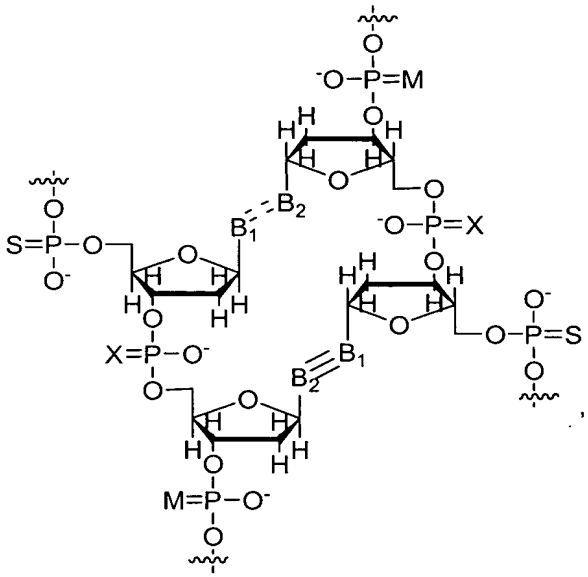
A. DESCRIPTION OF THE OLIGONUCLEOTIDES

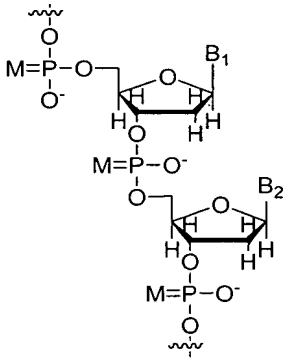
One of the features of the invention is the ability to provide improved nucleotide or oligonucleotide polymer conjugates. The polymer transport systems described herein are not limited to a single species of oligonucleotide but, instead, are designed to work with a wide variety of such moieties, it being understood that the polymer transport systems can attach to one or more of the 3'- or 5'- terminals, usually PO_4 or SO_4 groups of a nucleotide. The nucleotide sequences are depicted herein using conventional nomenclature, wherein the sequences are read from left to right, going from the 5'- terminus to the 3'-terminus (5'- $\rightarrow$ 3'-).

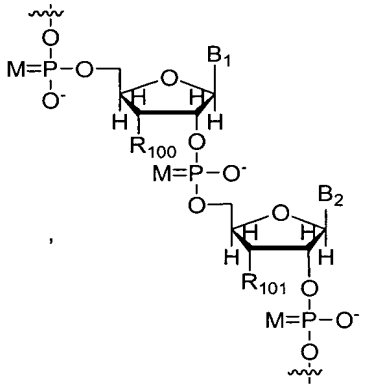
X_{1-3} represent the same or different nucleotide or oligonucleotide residue, which for purposes of the present invention include oligodeoxynucleotide residues. More preferably, X_{1-3} are independently selected antisense oligonucleotide residues or antisense oligodeoxynucleotide residues.

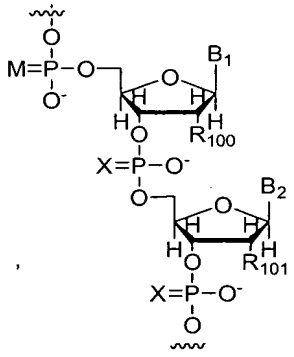
A non-limiting list of potential nucleotides which can be used either alone or as part of an oligonucleotide (10-1,000 nucleotides) include

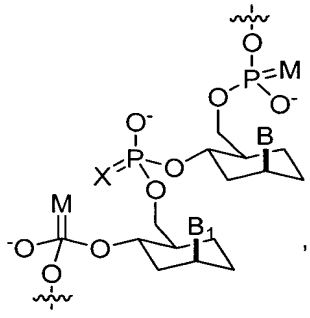


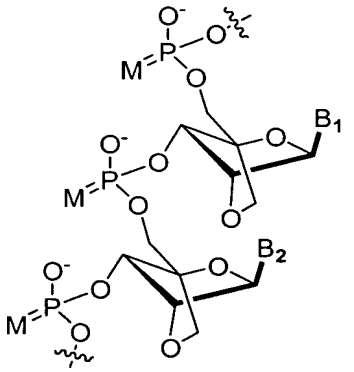


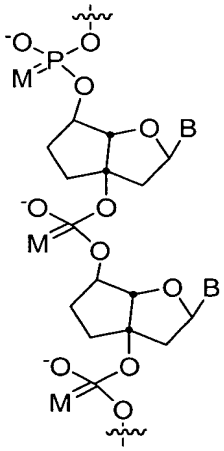












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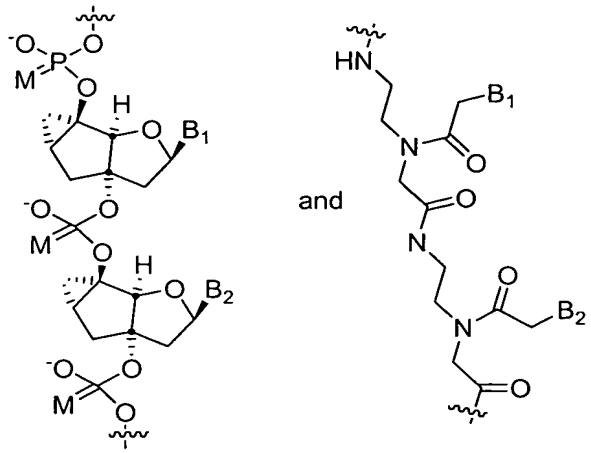


wherein

M is O or S;

B_1 and B_2 are independently selected from the group consisting of A (adenine), G (guanine), C (cytosine), T (thymine), U (uracil) and modified bases, including those shown below and those known to those of ordinary skill;

R_{100} and R_{101} are independently selected from the group consisting of H, OR' where R' is H, a C_{1-6} alkyl, substituted alkyl, nitro, halo, aryl, etc.

Some of the oligonucleotides and oligodeoxynucleotides useful in the methods of the invention include, but are not limited to, the following:

Oligonucleotides and oligodeoxynucleotides with natural phosphorodiester backbone or phosphorothioate backbone or any other modified backbone analogues;

LNA (Locked Nucleic Acid);

PNA (nucleic acid with peptide backbone);

tricyclo-DNA;

decoy ODN (double stranded oligonucleotide);

RNA (catalytic RNA sequence);

ribozymes;

spiegelmers (L-conformational oligonucleotides);

CpG oligomers, and the like, such as those disclosed at:

Tides 2002, Oligonucleotide and Peptide Technology Conferences, May 6-8, 2002, Las Vegas, NV and

Oligonucleotide & Peptide Technologies, 18th & 19th November 2003, Hamburg, Germany, the contents of which are incorporated herein by reference.

Oligonucleotides according to the invention can also optionally include any suitable art-known nucleotide analogs and derivatives, including those listed by Table 1, below.

TABLE 1
Representative Nucleotide Analogs And Derivatives

4-acetylcytidine	5-methoxyaminomethyl-2-thiouridine
5-(carboxyhydroxymethyl)uridine	beta, D-mannosylqueuosine
2'-O-methylcytidine	5-methoxycarbonylmethyl-2-thiouridine
5-carboxymethylaminomethyl-2-thiouridine	5-methoxycarbonylmethyluridine
5-carboxymethylaminomethyluridine	5-methoxyuridine
Dihydrouridine	2-methylthio-N6-isopentenyladenosine
2'-O-methylpseudouridine	N-((9-beta-D-ribofuranosyl-2-methylthiopurine-6-yl)carbamoyl)threonine
D-galactosylqueuosine	N-((9-beta-D-ribofuranosylpurine-6-yl)N-methylcarbamoyl)threonine
2'-O-methylguanosine	uridine-5-oxyacetic acid-methylester
Inosine	uridine-5-oxyacetic acid
N6-isopentenyladenosine	wybutoxosine
1-methyladenosine	pseudouridine
1-methylpseudouridine	queuosine
1-methylguanosine	2-thiocytidine
1-methylinosine	5-methyl-2-thiouridine
2,2-dimethylguanosine	2-thiouridine
2-methyladenosine	4-thiouridine
2-methylguanosine	5-methyluridine
3-methylcytidine	N-((9-beta-D-ribofuranosylpurine-6-yl)-carbamoyl)threonine
5-methylcytidine	2'-O-methyl-5-methyluridine
N6-methyladenosine	2'-O-methyluridine
7-methylguanosine	wybutosine
5-methylaminomethyluridine	3-(3-amino-3-carboxy-propyl)uridine

Preferably, the antisense oligonucleotide is one that downregulates a protein implicated in the resistance of tumor cells to anticancer therapeutics. For example, the protein BCL-2 inhibits the release of Cytochrome-C and Apoptosis Initiating Factor from mitochondria and thus prevents apoptosis from occurring. Cancer cells that have high levels of BCL-2 are thus very resistant to both chemotherapy or radiation therapy. U.S. Patent No. 6,414,134, incorporated by

reference herein, describes antisense oligonucleotides that downregulate the protein Bcl-2 that is associated with resistance to anticancer therapy in a number of tumor cells, *e.g.*, including prostate cancer cells, myeloma cells and other tumor cells. According to the above-noted U.S. patent, the bcl-2 gene is believed to contribute to the pathogenesis of cancer primarily by prolonging tumor cell survival rather than by accelerating cell division. U.S. Patent No. 6,414,134 generally describes antisense oligonucleotides of 17 to 35 bases in length, that are complementary to bcl-2 mRNA, and that include a nucleic acid molecule having the sequence of TACCGCGTGC GACCCTC (SEQ ID NO: 5). These preferably include at least one phosphorothioate linkage.

Other art-known cellular proteins that are contemplated by various companies as targets for downregulation by antisense oligonucleotides, for cancer therapy, are summarized by the following table.

Table 2

Antisense Agent	Target Protein
Affinitak (ISIS 3521)	PKC-alpha
ISIS 112989 (OGX 011)	Secretory Protein Clusterin
ISIS 23722	Survivin
AP 12009	TGF-Beta2
GEM 231	Protein kinase A
GEM 240	MDM2
IGF-1R/AS ODN	Insulin-like growth factor
MG98	DNA methyltransferase
LErafAON	C-raf-1
Ki-67 antisense oligonucleotide	Ki-67
GTI-2040	ribonucleotide reductase
ISIS 2503	H-ras
AP11014	TGF-Beta1

Antisense oligonucleotides suitable for use in downregulating the expression of proteins related to cancer cell survival, such as bcl-2 expression include oligonucleotides that are from about two to two hundred nucleotide codons; more preferably ten to forty codons; and most preferably about 17 to 20 codons. The oligonucleotides are preferably selected from those oligonucleotides complementary to strategic sites along the pre-mRNA of bcl-2, such as the

translation initiation site, donor and splicing sites, or sites for transportation or degradation.

Blocking translation at such strategic sites prevents formation of a functional bcl-2 gene product. It should be appreciated, however, that any combination or subcombination of anticode oligomers, including oligonucleotides complementary or substantially complementary to the bcl-2 pre-mRNA or mRNA that inhibit cell proliferation is suitable for use in the invention. For example, oligodeoxynucleotides complementary to sequence portions of contiguous or non-contiguous stretches of the bcl-2 RNA may inhibit cell proliferation, and would thus be suitable for use in methods of the invention.

Oligonucleotides suitable for downregulating bcl-2 expression also include oligonucleotides complementary or substantially complementary to sequence portions flanking the strategic or other sites along the bcl-2 mRNA. The flanking sequence portions preferably range from about two to about one hundred bases, upstream or downstream of the previously noted sites along the bcl-2 mRNA. These sites preferably range from about five to about twenty codons in length. It is also preferable that the oligonucleotides be complementary to a sequence portion of the pre-mRNA or mRNA that is not commonly found in pre-mRNA or mRNA of other genes, in order to minimize homology of the oligonucleotides for pre-mRNA or mRNA coding strands from other genes.

A number of preferred antisense, or complementary, oligonucleotides for downregulating bcl-2 are listed as follows by Table 3.

Table 3

translation initiation antisense (TI-AS)	3' . . . CCCTTCCTACCGCGTGCGAC . . . 5' (SEQ ID NO: 6)
bcl-2	5' . . . CTTTTCCTCTGGGAAGGATGGCGCACGCTGGGAGA . . . 3' (SEQ ID NO: 7)
splice donor antisense (SD-AS)	3' . . . CCTCCGACCCATCCACGTAG . . . 5' (SEQ ID NO: 8)
bcl-2	5' . . . ACGGGGTAC . . . GGAGGCTGGGTAGGTGCATCTGGT . . . 3' (SEQ ID NO: 9)

splice acceptor antisense (SA-AS)	3' . . . GTTGACGTCCTACGGAAACA . . . 5' (SEQ ID NO: 10)
bcl-2	5' . . . CCCCCAACTGCAGGATGCCTTTGTGGAACTGTACGG . . . 3' (SEQ ID NO: 11)

It will be appreciated that antisense oligonucleotides can be employed that comprise more or fewer substituent nucleotides, and/or that extend further along the bcl-2 mRNA chain in either the 3' or 5' direction relative to those listed by Table 3, *supra*.

Preferably, the antisense oligonucleotide employed in the prodrug of the invention has the same or substantially similar nucleotide sequence as does Genasense (a/k/a oblimersen sodium, produced by Genta Inc., Berkeley Heights, NJ). Genasense is an 18-mer phosphorothioate antisense oligonucleotide, TCTCCCAGCGTGCGCCAT (SEQ ID NO: 1), that is complementary to the first six codons of the initiating sequence of the human bcl-2 mRNA (human bcl-2 mRNA is art-known, and is described, *e.g.*, as SEQ ID NO: 19 in U.S. Patent No. 6,414,134, incorporated by reference herein). The U.S. Food and Drug Administration (FDA) has given Genasense Orphan Drug status in August 2000, and has accepted a New Drug Application (NDA) for Genasense in the treatment of cancer. The NDA proposes administering Genasense in combination with dacarbazine for the treatment of patients with advanced melanoma who have not previously received chemotherapy. In addition, the FDA granted Priority Review status to the application, which targets an agency action on or before June 8, 2004. See also, Chi *et al.*, 2001, *Clinical Cancer Research* Vol. 7, 3920-3927, incorporated by reference herein, confirming activity of Genasense in combination therapy of prostate cancer in early clinical trials. The prodrugs of the present invention have the same utility as that recognized for the native (unmodified) 18-mer.

Genasense has been shown to downregulate the production of the Bcl-2 protein and enhance a tumor cell's sensitivity to therapy and ultimately, cause cell death. A number of studies have reported promising results in treatment of several cancers with Genasense in combination with anticancer agents. A phase I/II trial of Genasense in combination with dacarbazine in patients with melanoma has

shown promising activity and a Phase III multicenter trial is under way. In addition, Genasense, used in combination with mitoxantrone in patients with hormone-refractory prostate cancer has shown promising results. Kim *et al.*, 2001, Id.

The conjugation of antisense oligonucleotides, such as Genasense, to polymers exemplifies one preferred embodiment of the invention.

In an alternative embodiment, additional suitable antisense oligonucleotides include:

T-C-T-C-C-C-A-G-C-G-T-G-C-G-C-C-A-T; (compound **13** – SEQ ID NO: 1)

T-C-T-C-C-C-A-G-C-A-T-G-T-G-C-C-A-T; (compound **36** – SEQ ID NO: 2)

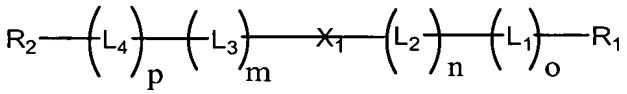
A-T-C-C-T-A-A-G-C-G-T-G-C-G-C-C-T-T; (compound **37** – SEQ ID NO: 3) and

T-C-T-C-C-C-A-G-X-G-T-G-X-G-C-C-A-T, (compound **38** – SEQ ID NO: 4)

as well as those found in the examples.

B. FORMULA (I)

In one preferred embodiment of the invention, there are provided oligonucleotide prodrugs of the formula (I):



(I)

wherein:

R_1 and R_2 are independently H or a polymer residue;

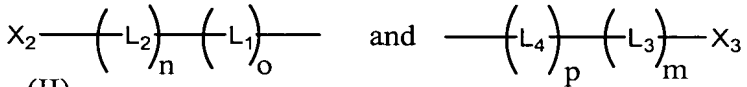
L_1 and L_4 are independently selected releasable linking moieties;

L_2 and L_3 are independently selected spacing groups;

X_1 is a nucleotide residue or an oligonucleotide residue;

m, n, o and p are independently zero or a positive integer, provided that either $(o + n)$ or $(p + m) \geq 2$.

The polymer transport system of the present invention is based in part on the least one of R_1 and R_2 preferably being a polymeric residue, optionally having a capping group designated herein as A. Suitable capping groups include, for example, OH, NH_2, SH, CO_2H, C_{1-6} alkyls, as well as oligonucleotide-containing groups such as



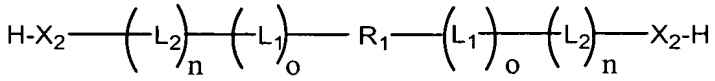
(II)

and

(III)

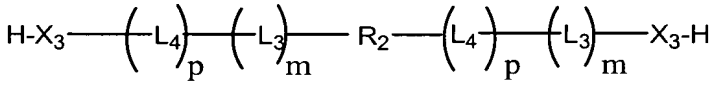
wherein X_2 and X_3 are either the same as X_1 or another nucleotide or oligonucleotide residue.

The preferred capping groups (II) and (III) allow compositions of formulas (i), (ii), (iii) and (iv) shown below to be formed:



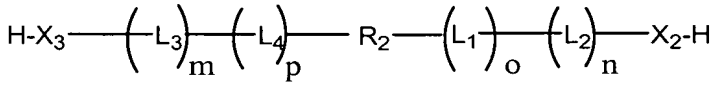
(i)

bis-3'-oligonucleotide,



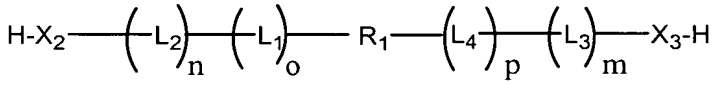
(ii)

bis-5'-oligonucleotide,



(iii)

bis-5', 3'-oligonucleotide, and

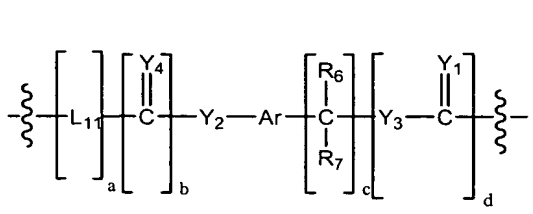


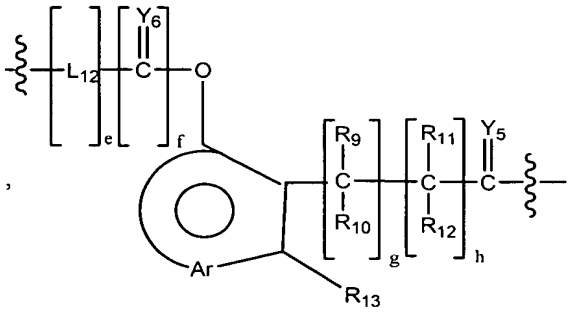
(iv)

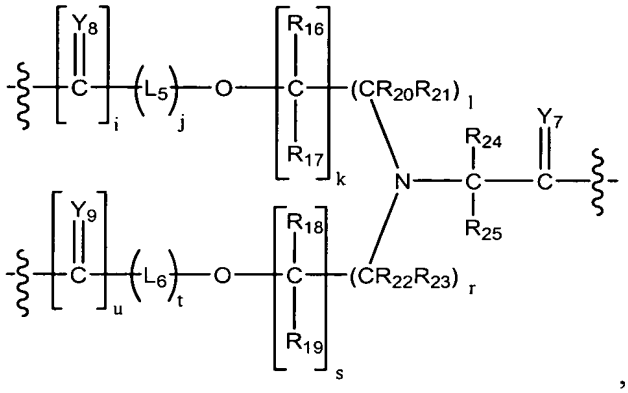
bis-3', 5'-oligonucleotide,

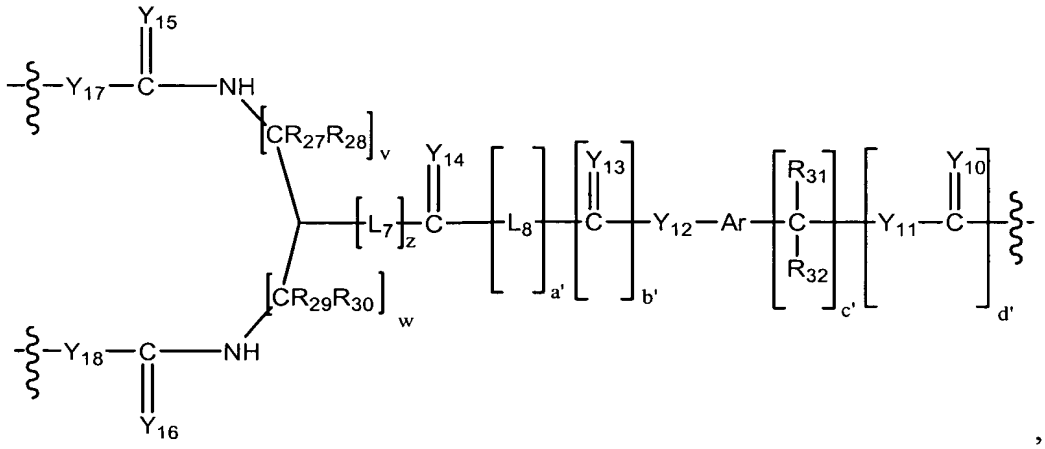
wherein all variables are as previously described.

In another preferred embodiment of the invention, L_4 is a releasable linking moiety selected from among the formulas:

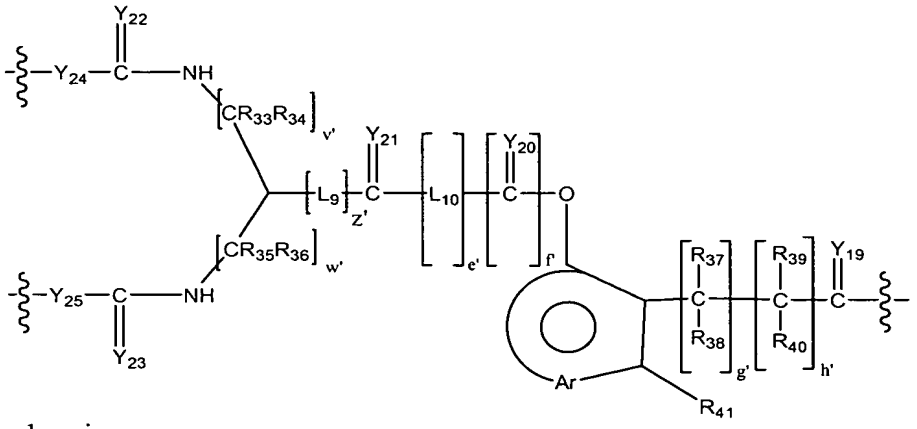








and



wherein:

Y_{1-25} are independently selected from the group consisting of O, S or NR_9;

R_{6-7}, R_{9-13}, R_{16-25}, and R_{27-41} are independently selected from the group consisting of hydrogen, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cyloalkyls, aryls, substituted aryls, aralkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy and C_{1-6} heteroalkoxy;

Ar is a moiety which forms a multi-substituted aromatic hydrocarbon or a multi-substituted heterocyclic group;

L_{5-12} are independently bifunctional spacers;

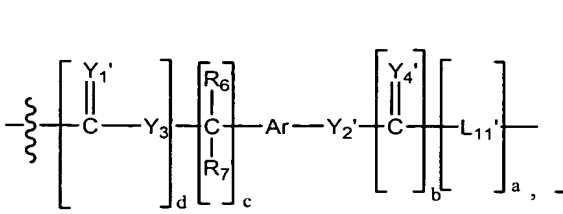
Z is selected from among moieties actively transported into a target cell, hydrophobic moieties, bifunctional linking moieties and combinations thereof;

c, h, k, l, r, s, v, w, v', w', c', and h' are independently selected positive integers;

a, e, g, j, t, z, a', z', e' and g' are independently either zero or a positive integer; and

b, d, f, i, u, q, b', d' and f' are independently zero or one.

In another preferred embodiment L_1 is a releasable linking moiety selected from among the formulas: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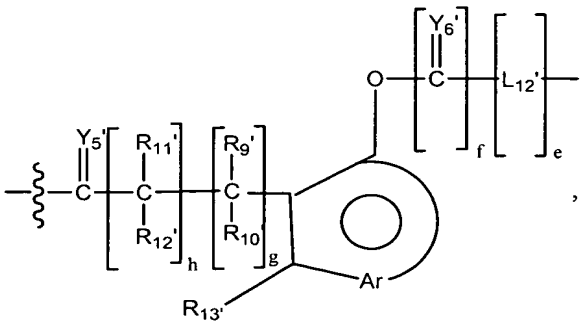
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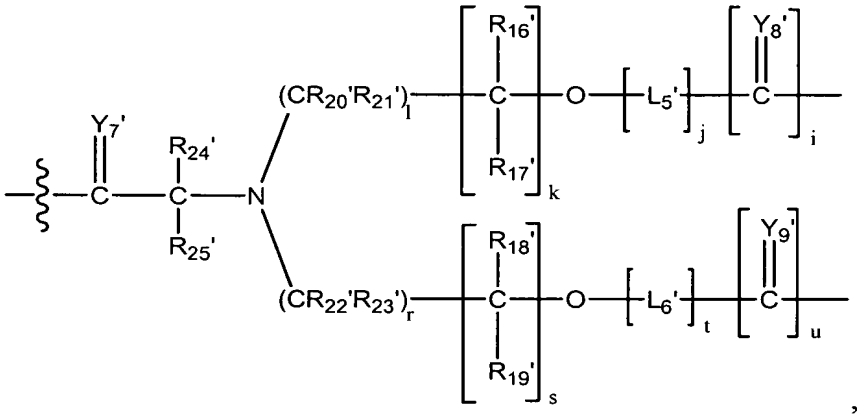
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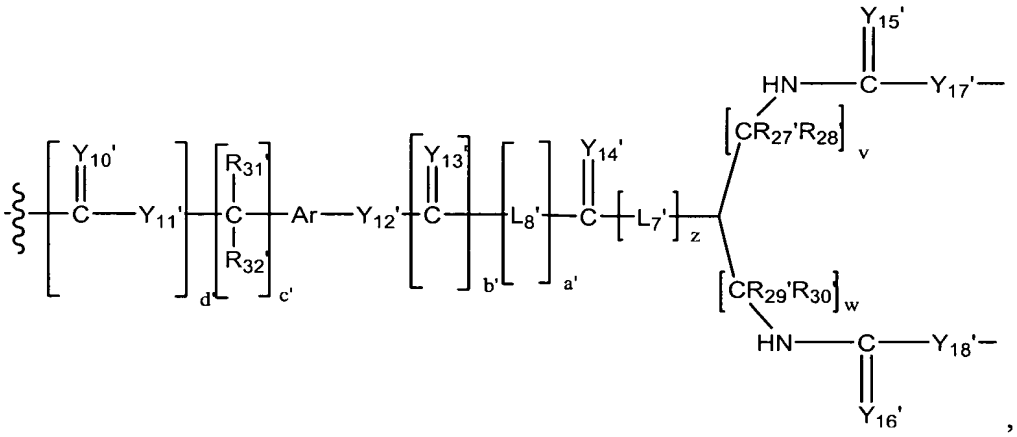
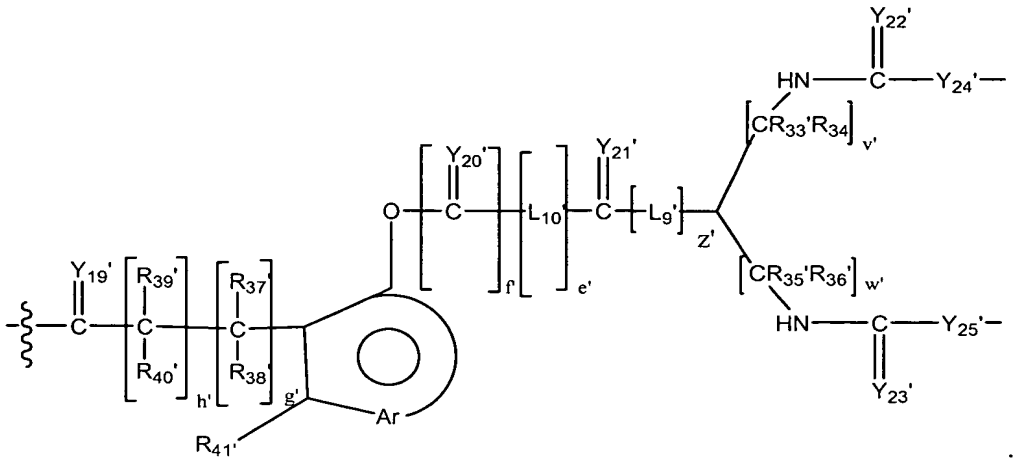

,

and



wherein

$Y_{1'-Y_{25'}}$ are independently selected from the group consisting of O, S or NR_9;

$R_{6'-7'}$, $R_{9'-13'}$, $R_{16'-25'}$, and $R_{27'-41'}$ are independently selected from the group consisting of hydrogen, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cyloalkyls, aryls, substituted aryls, aralkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy and C_{1-6} heteroalkoxy; and

$L_{5'-12'}$ are independently bifunctional spacers.

In some preferred embodiments of the invention, L_{5-12} are independently bifunctional spacers selected from among:

$-(CH_2)_3-$,

$-C(O)NH(CH_2)_3-$,

$-NH\ (CH_2)_3-$,

$-(CR_{55}R_{56})_sO(CR_{57}R_{58})_tC(O)-$

$-NR_{59}(CH_2)_r(OCH_2CH_2)_rNH-$

$-(OCH_2CH_2)_sNH-$

$-O(CR_{55}R_{56})_sNH-$

$-NR_{59}(CR_{57}R_{58})_tC(O)NH(CR_{55}R_{56})_sC(O)-$

$-O(CH_2)_sOC(O)-$

$-NR_{59}(CR_{55}R_{56})_sC(O)-$

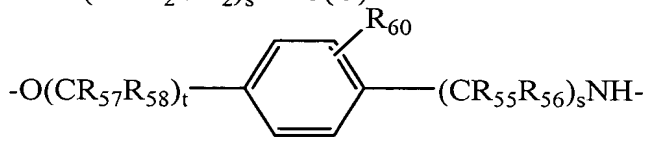
$-NR_{59}(CH_2)_t(OCH_2CH_2)_sNHC(O)-$

$-NR_{59}(OCH_2CH_2)_sOC(O)-$

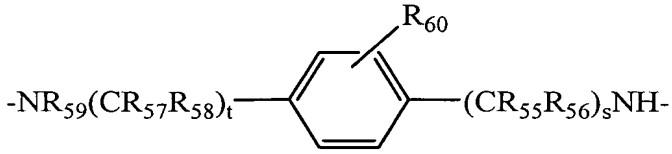
$-O(CR_{55}R_{56})_sNHC(O)-$

$-O(CR_{55}R_{56})_sOC(O)-$

$(OCH_2CH_2)_sNHC(O)-$



and



and $L_{5'-12'}$ are independently bifunctional spacers selected from among:

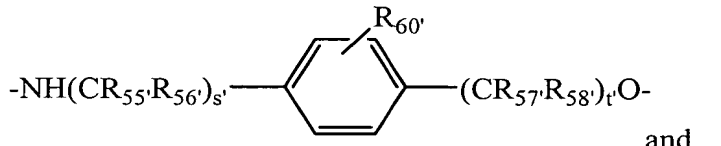
$-(CH_2)_3-$,

$-(CH_2)_3NH-C(O)$,

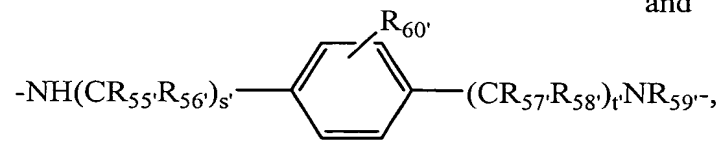
$-(CH_2)_3NH-$,

$-C(O)(CR_{57'}R_{58'})_{s'}O(CR_{55'}R_{56'})_{t'}$,

$-NH(CH_2CH_2O)_{s'}(CH_2)_{t'}NR_{59'}-$,
$-NH(CH_2CH_2O)_{s'}-$,
$-NH(CR_{55'}R_{56'})_{s'}O-$,
$-C(O)(CR_{55'}R_{56'})_{s'}NHC(O)(CR_{57'}R_{58'})_{t'}NR_{59'}-$,
$-C(O)O(CH_2)_{s'}O-$,
$-C(O)(CR_{55'}R_{56'})_{s'}NR_{59'}-$,
$-C(O)NH(CH_2CH_2O)_{s'}(CH_2)_{t'}NR_{59'}-$,
$-C(O)O-(CH_2CH_2O)_{s'}NR_{59'}-$,
$-C(O)NH(CR_{55'}R_{56'})_{s'}O-$,
$-C(O)O(CR_{55'}R_{56'})_{s'}O-$,
$-C(O)NH(CH_2CH_2O)_{s'}-$,



and



wherein:

R_{55}-R_{59} and $R_{55'}$-$_{59'}$ are independently selected from the group consisting of hydrogen, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cyloalkyls, aryls substituted aryls, aralkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy and C_{1-6} heteroalkoxy, and

R_{60} and $R_{60'}$ is selected from the group consisting of hydrogen, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cyloalkyls, aryls substituted aryls, aralkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy, C_{1-6} heteroalkoxy, NO_2, haloalkyl and halogen; and

s' and t' are each a positive integer.

In another preferred embodiment of the invention, L_2 and L_3 are independently spacing groups having about 1 to about 60 carbon atoms and from about 1 to about 10 heteroatoms. Preferably, L_2 and L_3 are independently spacing

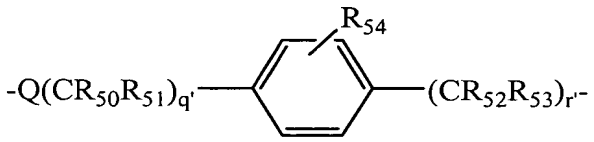
groups having from about 2 to about 10 carbon atoms and from about 1 to about 6 heteroatoms. Most preferably, L_3 is selected from among:

$-Q(CR_{50}R_{51})_{q'}-$,

$-Q(CR_{50}R_{51})q'O(CR_{52}R_{53})r'$

$-Q(CH_2CH_2O)_{q'}(CR_{52}R_{53})_{r'}-$,

$-QCR_{50}R_{51})_{q'}NHC(O)(CR_{52}R_{53})_{r'}-$,



and

$-Q(CH_2)_{q'}-S-S-(CH_2)_{r'}-$; and

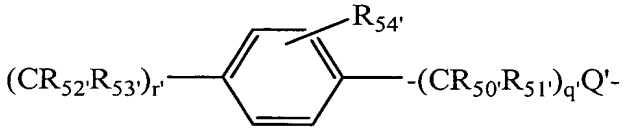
most preferably, L_2 is selected from among:

$-(CR_{50'}R_{51'})_{q'}Q'-$,

$-(CR_{52'}R_{53'})_{r'}O(CR_{50'}R_{51'})_{q'}Q'-$

$-(CR_{52'}R_{53'})_{r'}(OCH_2CH_2)Q'-$,

$-(CR_{52'}R_{53'})_{r'}C(O)NH(CR_{50'}R_{51'})_{q'}Q'-$,



and

$-(CH_2)-S-S-(CH_2)_{q'}Q-$

wherein.

Q and Q' are independently selected from O, S or NH;

R_{50-53} and $R_{50'-53'}$ are independently selected from the group consisting of hydrogen, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cyloalkyls, aryls substituted aryls, aralkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy and C_{1-6} heteroalkoxy;

R_{54} and $R_{54'}$ are independently selected from the group consisting of hydrogen, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cyloalkyls, aryls substituted aryls, aralkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy, C_{1-6} heteroalkoxy, NO_2, haloalkyl and halogen; and

q' and r' are each a positive integer.

With regard to the other variables which comprise the formulae of the present invention, the following are preferred:

Y_{1-25} and $Y_{1'-25'}$ are independently selected from the group consisting of O, S or NR_9;

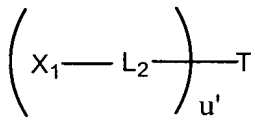
R_{6-7}, R_{9-13}, R_{16-25}, R_{27-41}, and $R_{6'-7'}$, $R_{9'-13'}$, $R_{16'-25'}$, and $R_{27'-41'}$ are independently selected from the group consisting of hydrogen, C_{1-6} alkyls, C_{3-8} cycloalkyls, aryls, aralkyls, and C_{1-6} heteroalkyls;

c, h, k, l, r, s, v, w, v', w', c', and h' are one;

a, e, g, j, t, z, a', z', e' and g' are independently either zero or one; and

b, d, f, i, u, q, b', d' and f' are independently zero or one.

In yet another preferred embodiment of the invention there are provided compounds of the formula (Ia):



(v)

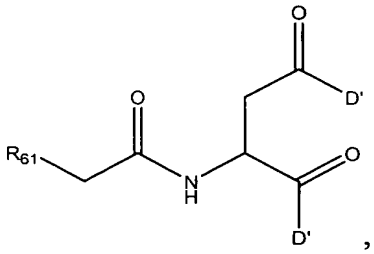
wherein:

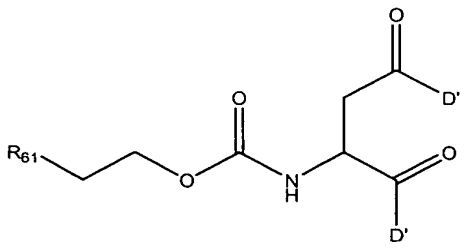
L_2 is a spacing group;

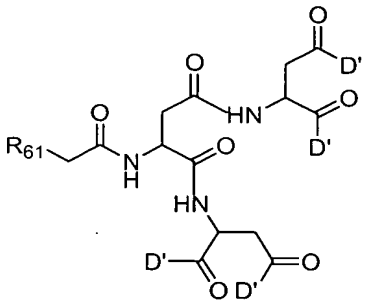
X_1 is a nucleotide or an oligonucleotide residue;

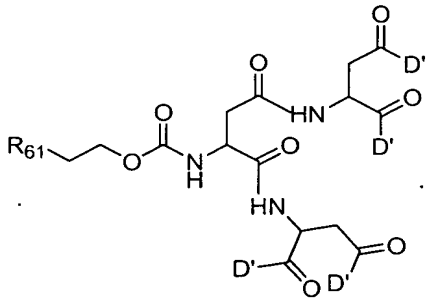
u' is a positive integer; and

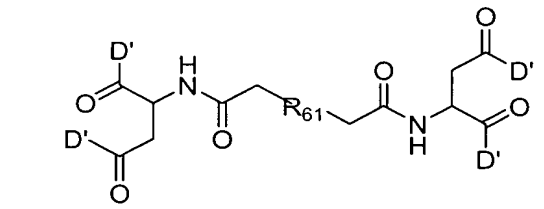
T is a branched polymer which is preferably selected from among those compounds described in commonly assigned PCT publication numbers WO02/065988 and WO02/066066, the disclosure of each being incorporated herein by reference. Within these general formulae, the following are preferred:

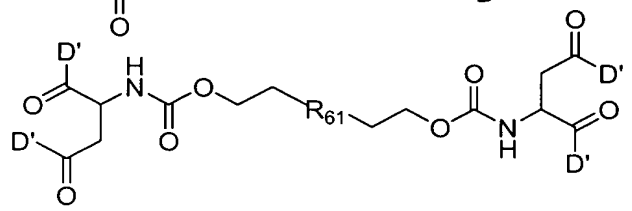


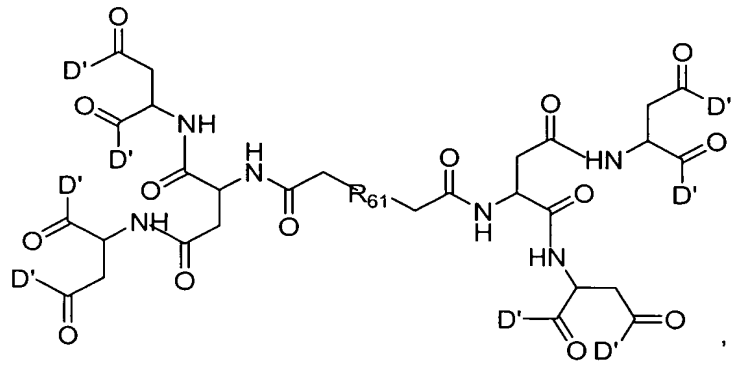


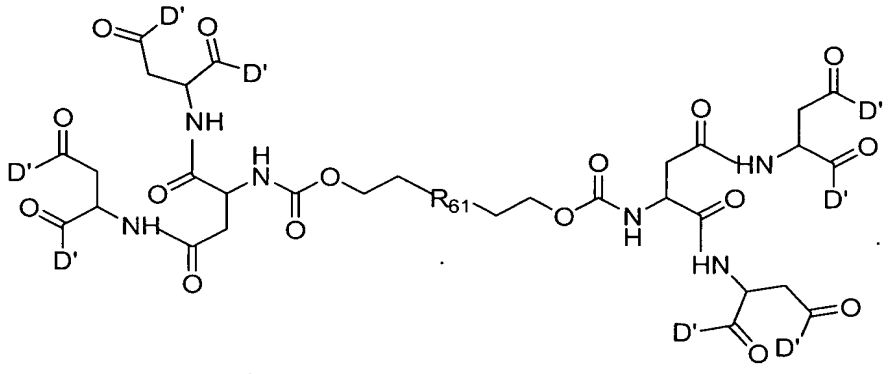


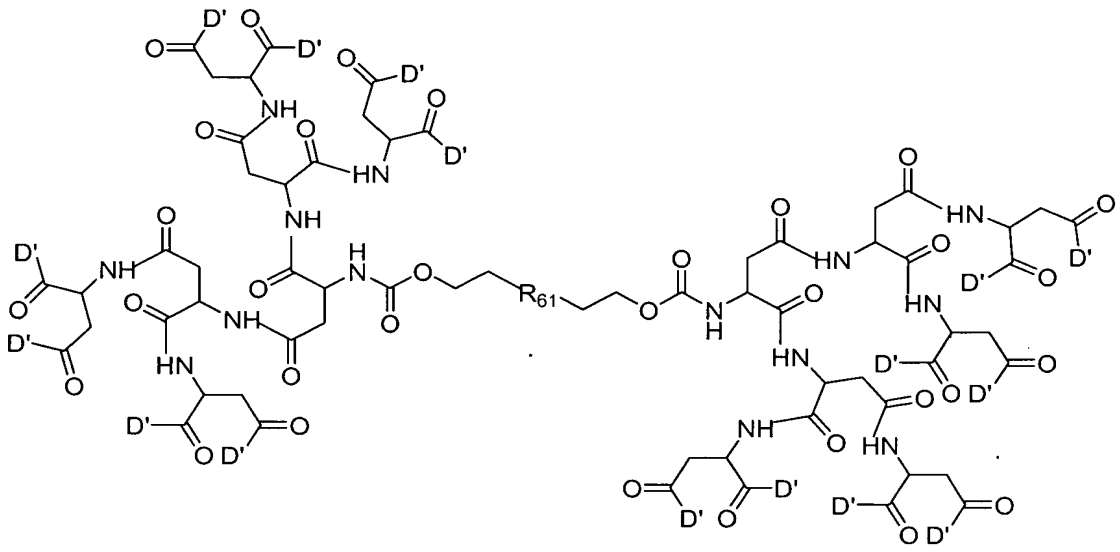




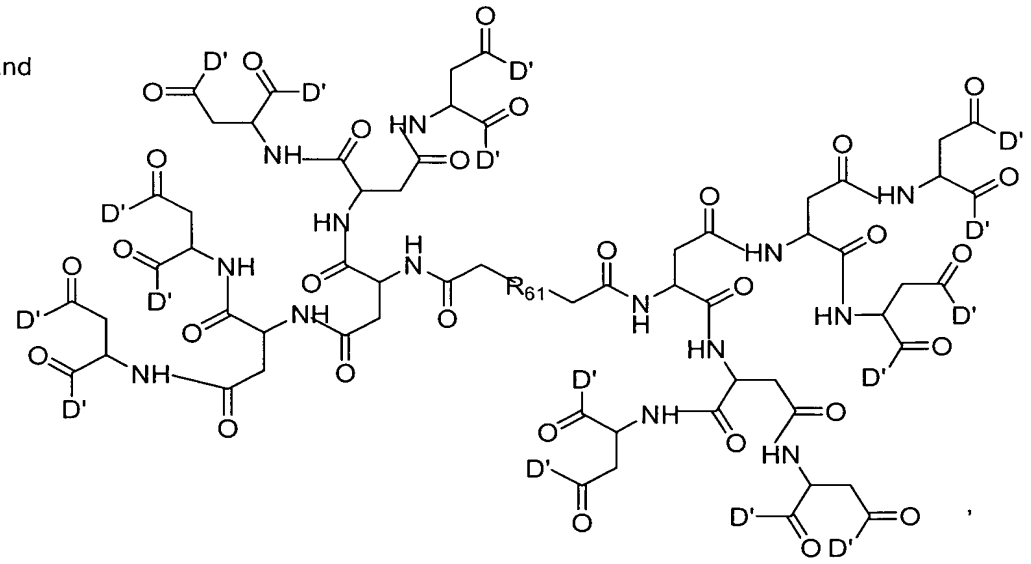






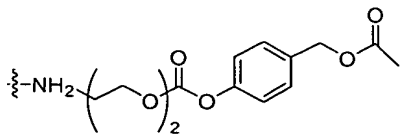


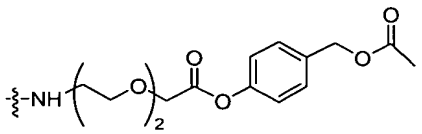
and

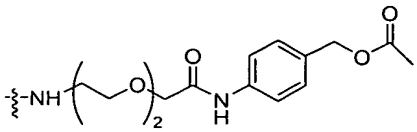


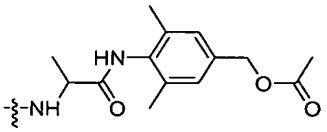
wherein:

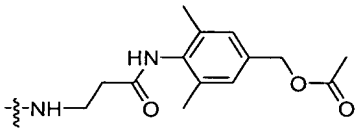
D' is one of

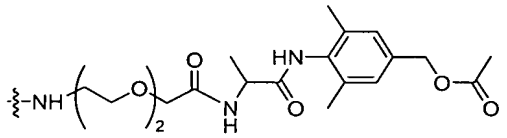


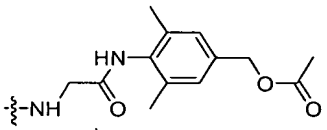


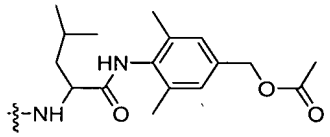


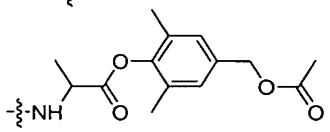


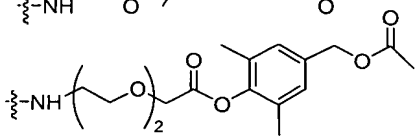


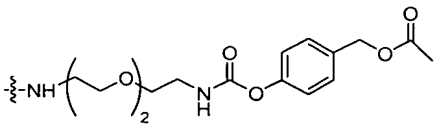


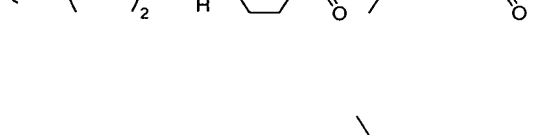


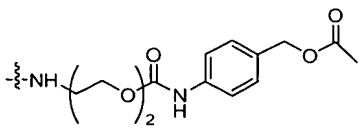


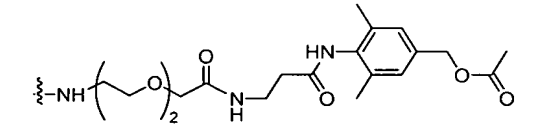


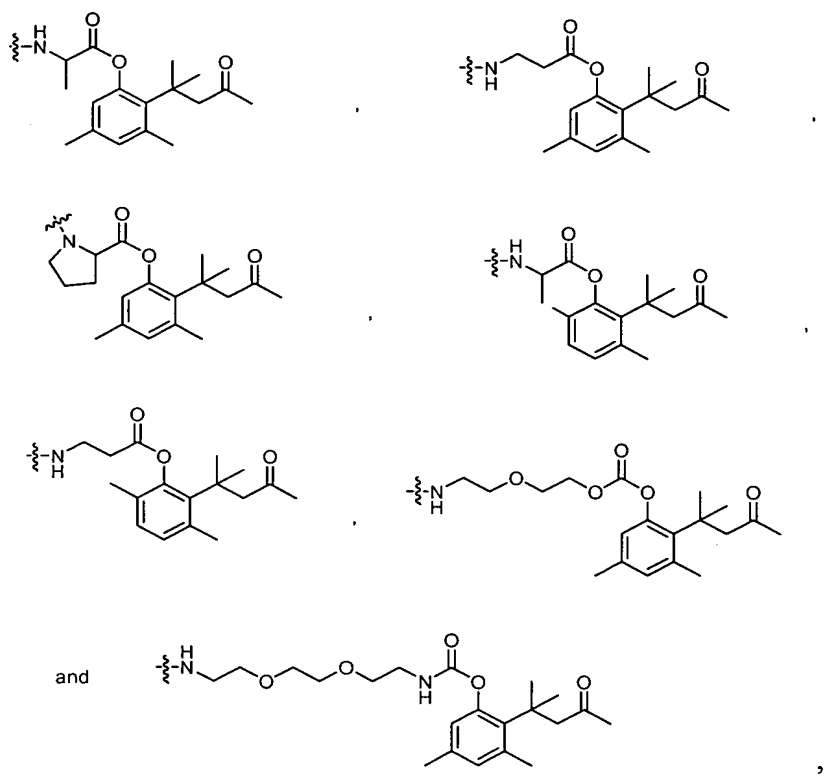








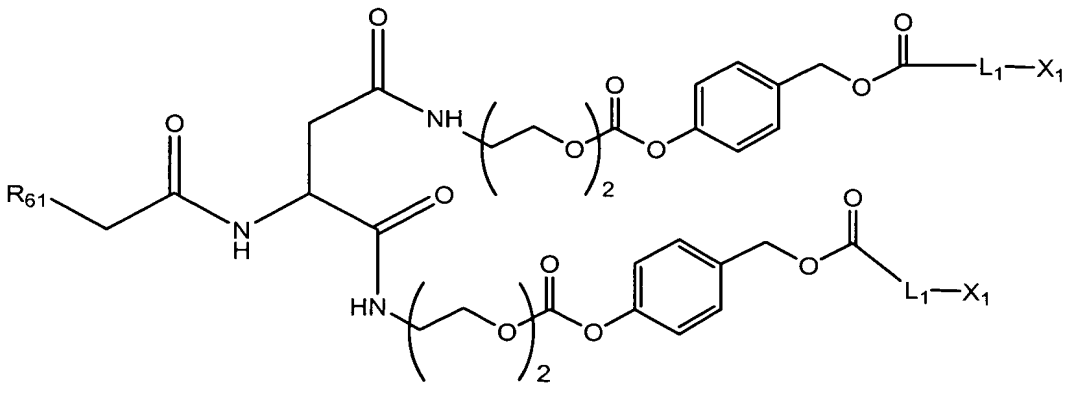




and

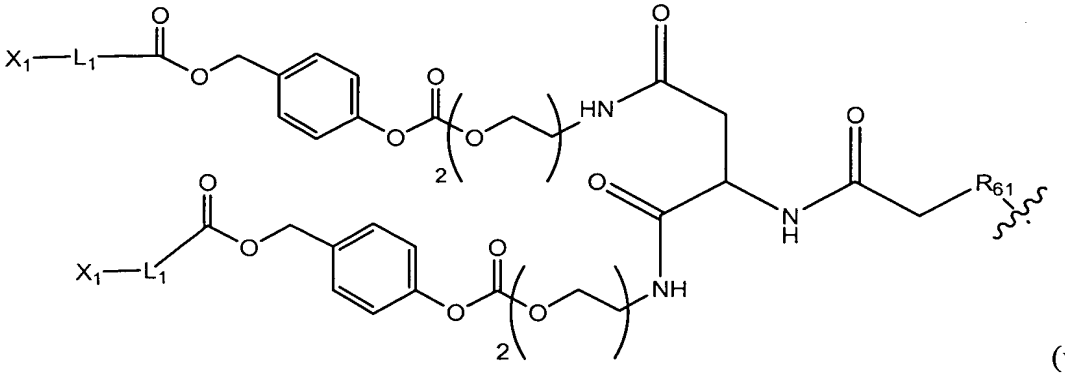
wherein R_{61} is a polymer residue such as that defined for R_1 with the understanding that the polymer can be bifunctional when R_{61} is shown with substitutions on both termini; and all other variables are as described above.

For illustrative purposes, a non-limiting compound of formula (Ia) is:

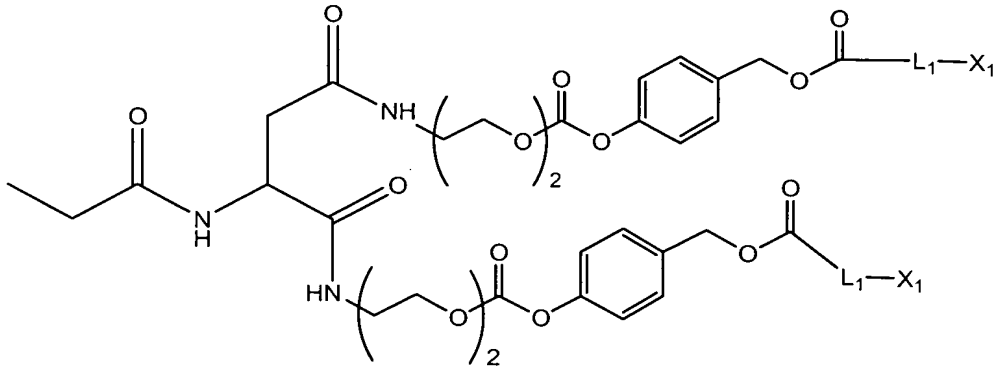


wherein all variables are as described above.

Another aspect of formula (Ia) includes bifunctional compounds that are formed when the polymeric residue (R_{61}) includes both an alpha and an omega terminal linking group so that at least four oligonucleotides are delivered. Examples of such polymer conjugates are illustrated below as formulas (vi) and (vii):



(vi)



(vii)

wherein all variables are as described above.

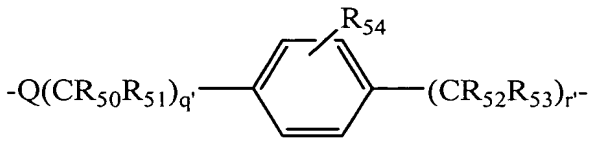
In another preferred embodiment of the invention, L_2 and L_3 are independently spacing groups having about 1 to about 60 carbon atoms and from about 1 to about 10 heteroatoms. Preferably, L_2 and L_3 are independently spacing groups having from about 2 to about 10 carbon atoms and from about 1 to about 6 heteroatoms. Most preferably, L_3 is selected from among:

$-Q(CR_{50}R_{51})_{q'}-$,

$-Q(CR_{50}R_{51})q'O(CR_{52}R_{53})r'$

$-Q(CH_2CH_2O)_{q'}(CR_{52}R_{53})_{r'}-$,

$-QCR_{50}R_{51})_{q'}NHC(O)(CR_{52}R_{53})_{r'}-$,



and

$-Q(CH_2)_{q'}-S-S-(CH_2)_{r'}-$, and

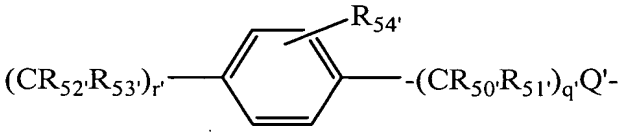
most preferably, L_2 is selected from among:

$-(CR_{50'}R_{51'})_{q'}Q'-$,

$-(CR_{52'}R_{53'})_{r'}O(CR_{50'}R_{51'})_{q'}Q'-$

$-(CR_{52'}R_{53'})_{r'}(OCH_2CH_2)Q'-$,

$-(CR_{52'}R_{53'})_{r'}C(O)NH(CR_{50'}R_{51'})_{q'}Q'-$,



and

$-(CH_2)-S-S-(CH_2)_{q'}Q-$

wherein.

Q and Q' are independently selected from O, S or NH;

R_{50-53} and $R_{50'-53'}$ are independently selected from the group consisting of hydrogen, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cyloalkyls, aryls substituted aryls, aralkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy and C_{1-6} heteroalkoxy;

R_{54} and $R_{54'}$ are independently selected from the group consisting of hydrogen, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cyloalkyls, aryls substituted aryls, aralkyls, C_{1-6} hetero-

alkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy, C_{1-6} heteroalkoxy, NO_2, haloalkyl and halogen; and

q' and r' are each a positive integer.

With regard to the other variables which comprise the formulae of the present invention, the following are preferred:

Y_{1-25} and $Y_{1'-25'}$ are independently selected from the group consisting of O, S or NR_9;

R_{6-7}, R_{9-13}, R_{16-25}, R_{27-41}, and $R_{6'-7'}$, $R_{9'-13'}$, $R_{16'-25'}$, and $R_{27'-41'}$ are independently selected from the group consisting of hydrogen, C_{1-6} alkyls, C_{3-8} cycloalkyls, aryls, aralkyls, and C_{1-6} heteroalkyls;

c, h, k, l, r, s, v, w, v', w', c', and h' are one;

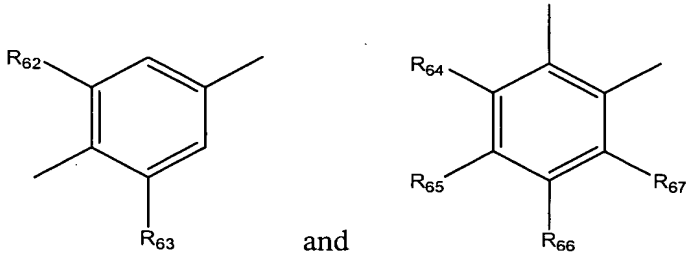
a, e, g, j, t, z, a', z', e' and g' are independently either zero or one; and

b, d, f, i, u, q, b', d' and f' are independently zero or one.

C. DESCRIPTION OF THE Ar MOIETY

In certain aspects of the invention, it can be seen that the Ar moiety is a moiety which when included in Formula (I) forms a multi-substituted aromatic hydrocarbon or a multi-substituted heterocyclic group. A key feature is that the Ar moiety is aromatic in nature. Generally, to be aromatic, the *pi* electrons must be shared within a "cloud" both above and below the plane of a cyclic molecule. Furthermore, the number of *pi* electrons must satisfy the Hückel rule (4n+2). Those of ordinary skill will realize that a myriad of moieties will satisfy the aromatic requirement of the moiety for formula (I) and thus are suitable for use herein.

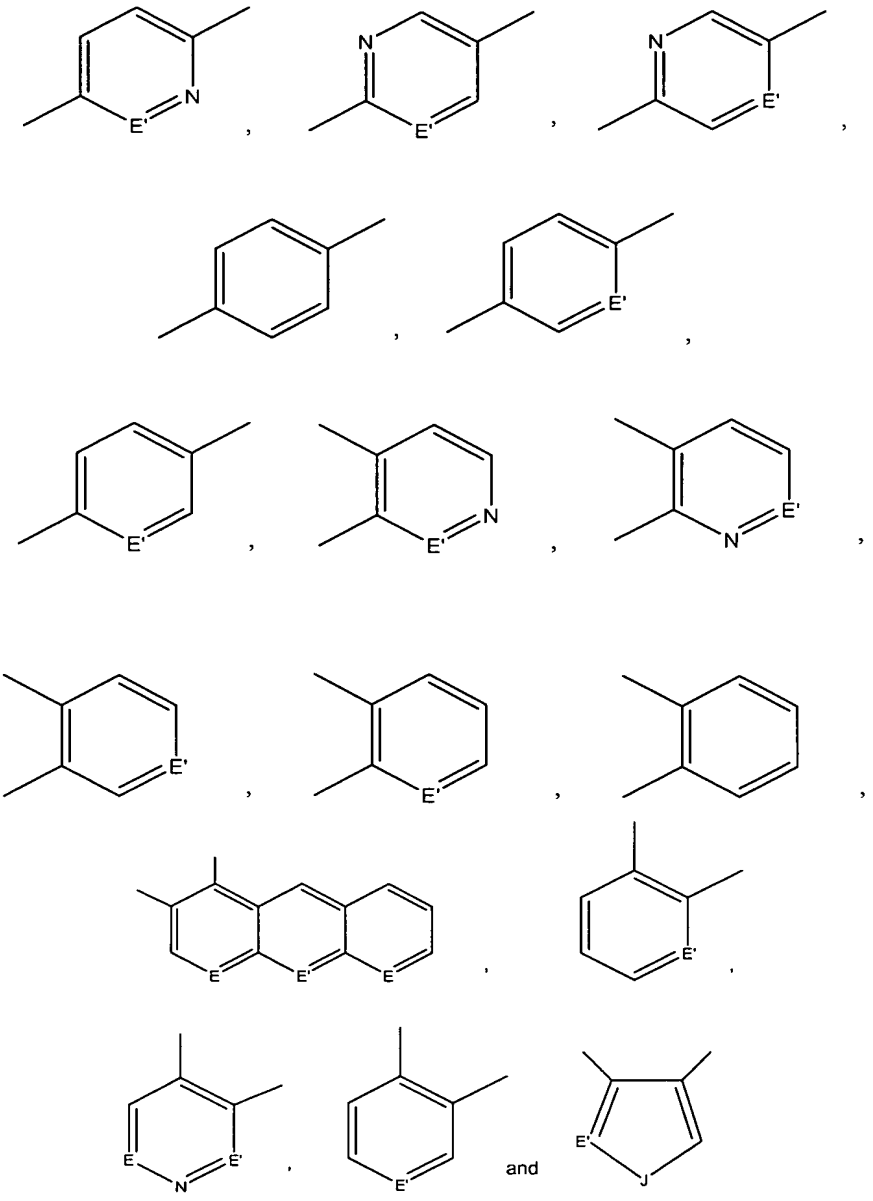
Some particularly preferred aromatic groups include:



and

wherein R_{62-67} are independently selected from the same group which defines R_6.

Other preferred aromatic hydrocarbon moieties include, without limitation



wherein E and E' are independently CR_{68} or NR_{69}; and J is O, S or NR_{70} where R_{68-70} are selected from the same group at that which defines R_6 or a cyano, nitro, carboxyl, acyl, substituted acyl or carboxyalkyl. Isomers of the five and six-membered rings are also contemplated as well as benzo- and dibenzo- systems and their related congeners are also contemplated. It will also be appreciated by the artisan of ordinary skill that aromatic rings can optionally be substituted with hetero-atoms such as O, S, NR_9, etc. so long as Hückel's rule is obeyed.

Furthermore, the aromatic or heterocyclic structures may optionally be substituted with halogen(s) and/or side chains as those terms are commonly understood in the art.

D. POLYALKYLENE OXIDES

Referring to Formula (I) it can be seen that R_1 and R_2 are polymer moieties such as polyalkylene oxide. Suitable examples of such polymers include polyethylene glycols which are substantially non-antigenic. Also useful are polypropylene glycols, such as those described in commonly-assigned U.S. Pat. Nos. 5,643,575, 5,919,455 and 6,113,906. Other PEG's useful in the methods of the invention are described in Shearwater Polymers, Inc. catalog "Polyethylene Glycol and Derivatives 2001". The disclosure of each is incorporated herein by reference. R_1 and R_2 are preferably PEG derivatives, e.g. - $O\text{-}(CH_2CH_2O)_x$ – Within this aspect, $R_{1\text{-}2}$ are independently selected from among:

$$J\text{- }O\text{-}(CH_2CH_2O)_{n'}\text{-}$$
$$J\text{-}O\text{-}(CH_2CH_2O)_{n'}\text{-}CH_2C(O)\text{-}O\text{-},$$
$$J\text{-}O\text{-}(CH_2CH_2O)_{n'}\text{-}CH_2CH_2\ NR_{48}\text{-},$$
$$J\text{-}O\text{-}(CH_2CH_2O)_{n'}\text{-}CH_2CH_2\ SH,$$
$$\text{-}O\text{-}C(O)CH_2\text{-}O\text{-}(CH_2CH_2O)_{n'}\text{-}CH_2C(O)\text{-}O\text{-},$$
$$\text{-}NR_{48}CH_2CH_2\text{-}O\text{-}(CH_2CH_2O)_{n'}\text{-}CH_2CH_2\ NR_{48}\text{-},$$
$$\text{-}SHCH_2CH_2\text{-}O\text{-}(CH_2CH_2O)_{n'}\text{-}CH_2CH_2\ SH\text{-},$$

wherein

n' is the degree of polymerization selected so that the weight average molecular weight is at least about 2,000 Da to about 136,000 Da;

R_{48} is selected from the group consisting of hydrogen, $C_{1\text{-}6}$ alkyls, $C_{3\text{-}12}$ branched alkyls, $C_{3\text{-}8}$ cycloalkyls, $C_{1\text{-}6}$ substituted alkyls, $C_{3\text{-}8}$ substituted cyloalkyls, aryls substituted aryls, aralkyls, $C_{1\text{-}6}$ heteroalkyls, substituted $C_{1\text{-}6}$ heteroalkyls, $C_{1\text{-}6}$ alkoxy, phenoxy and $C_{1\text{-}6}$ heteroalkoxy; and

J is a capping group such as methyl or a complementary linking group which allows a bifunctional polymer to be provided.

Although PAO's and PEG's can vary substantially in weight average molecular weight, preferably, R_1 and R_2 independently have a weight average molecular weight of from about 2,000 Da to about 136,000 Da in most aspects of

the invention. More preferably, R_1 and R_2 independently have a weight average molecular weight of from about 3,000 Da to about 100,000 Da, with a weight average molecular weight of from about 5,000 Da to about 40,000 Da being most preferred.

The polymeric substances included herein are preferably water-soluble at room temperature. A non-limiting list of such polymers include polyalkylene oxide homopolymers such as polyethylene glycol (PEG) or polypropylene glycols, polyoxyethylenated polyols, copolymers thereof and block copolymers thereof, provided that the water solubility of the block copolymers is maintained.

E SYNTHESIS OF OLIGONUCLEOTIDE POLYMER CONJUGATES

Generally the prodrugs are prepared by

a) reacting a compound of the formula:

$$R_2\text{-}L_4\text{-leaving group}$$

with a compound of the formula:

$$H\text{-}L_3\text{-}X_1$$

under conditions sufficient to form a prodrug of the formula

$$R_2\text{-}L_4\text{-}L_3\text{-}X_1 ,$$

wherein:

R_2 is a polymer residue;

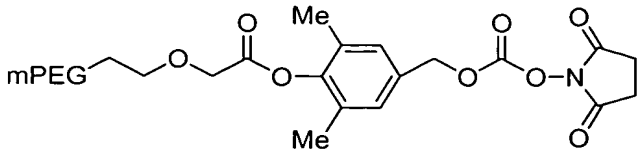
L_4 is a releasable linking moiety;

L_3 is a spacing group;

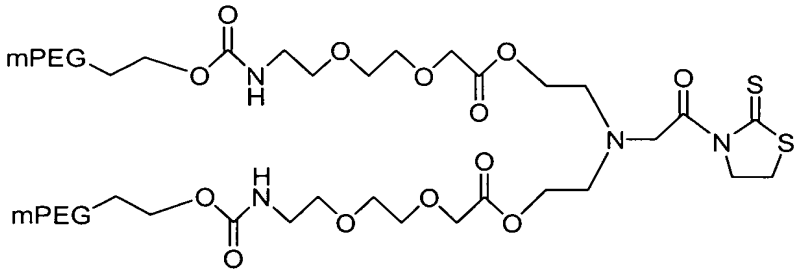
X_1 is a nucleotide or an oligonucleotide residue.

Within this aspect of the invention, it is preferred to employ activated polymers which already contain the releasable linkers attached thereto. A non-limiting list of suitable combinations include the releasable PEG-based transport systems described in commonly-assigned U.S. Patent Nos. 6,624,142, 6,303,569, 5,965,119, 6,566,506. 5,965,119, 6,303,569, 6,624,142, and 6,180,095. the contents of each are incorporated herein by reference.

Specific examples include but are not limited to,



1 (PEG mw 12,000) , and



8 (PEG mw 12,000) ,

it being understood of course that the molecular weight of the polymer portion can be varied according to the needs of the artisan.

The polymer releasable linker shown above is then reacted with a modified oligomer under conditions sufficient to allow the conjugate to be formed.

Any of the nucleotides or oligonucleotides described above can be functionalized on one of the 5'- or 3'- terminal phosphate or phosphorothioate using routine techniques such as the phosphoramidite methods to attach a desired alkyl-amino or other group onto the terminal phosphate. For example, a blocked (Fmoc) amino alkyl is attached, the resultant compound is oxidized, deprotected and purified.

Synthesis of specific oligonucleotide polymer conjugates or prodrugs is set forth in the Examples. Alternatively, the prodrugs can be prepared by:

1) reacting an activated PEG polymer with a protected bifunctional releasable linking group under suitable coupling conditions to form a first intermediate,

2) deprotecting and activating the intermediate of step 1) with a suitable activating group such as NHS ester, and

3) reacting the activated intermediate of step 2) with a modified oligonucleotide in a PBS buffered system to obtain the desired oligonucleotide polymer prodrug.

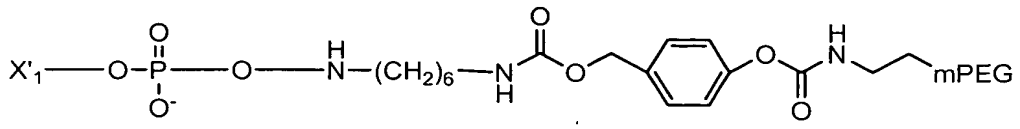
A non-limiting list of activated polymers include bis-succinimidyl carbonate activated PEG (**SC-PEG**), bis-thiazolidine-2-thione activated PEG (**T-PEG**), N-hydroxyphthalamidyl carbonate activated PEG (**BSC-PEG**),(see commonly assigned USSN 09/823,296, the disclosure of which is incorporated herein by reference), succinimidyl succinate activated PEG (**SS-PEG**) and mono-activated PEG's such as those found in, for example, in the aforementioned 2001 Shearwater Catalog.

Conjugation of the activated PEG polymer to the protected bifunctional releasable linking group can be carried out in the presence of a coupling agent. A non-limiting list of suitable coupling agents include 1,3-diisopropylcarbodiimide (DIPC), any suitable dialkyl carbodiimide, 2-halo-1-alkyl-pyridinium halides (Mukaiyama reagents), 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (EDC), propane phosphonic acid cyclic anhydride (PPACA) and phenyl dichlorophosphates, etc. which are available, for example from commercial sources such as Sigma-Aldrich Chemical, or synthesized using known techniques.

Preferably the substituents are reacted in an inert solvent such as tetrahydrofuran (THF), acetonitrile (CH_3CN), methylene chloride (DCM), chloroform ($CHCl_3$), dimethyl formamide (DMF) or mixtures thereof, and at a temperature from 0 °C up to about 22 °C (room temperature).

Conjugation of the modified oligonucleotide to the PEG-releasable linker can be carried out in a PBS buffered system in the pH range of about 7.4-8.5. The artisan of course will appreciate that synthesis of the prodrugs described herein, will also include the use of commonly found laboratory conditions, *i.e.* solvents, temperature, coupling agents etc. such as those described in the examples.

Regardless of the synthesis selected, some of the preferred compounds which result from the synthetic techniques described herein include:



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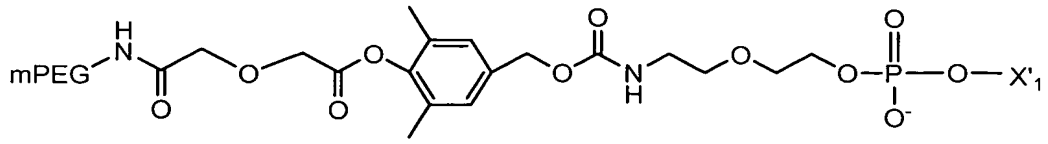

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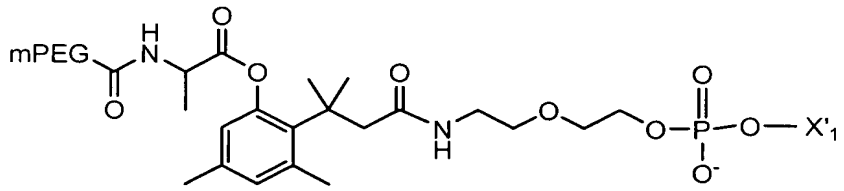

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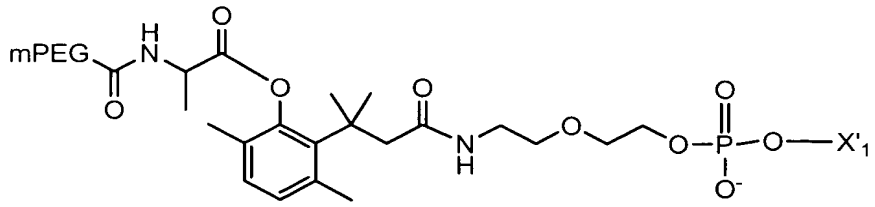

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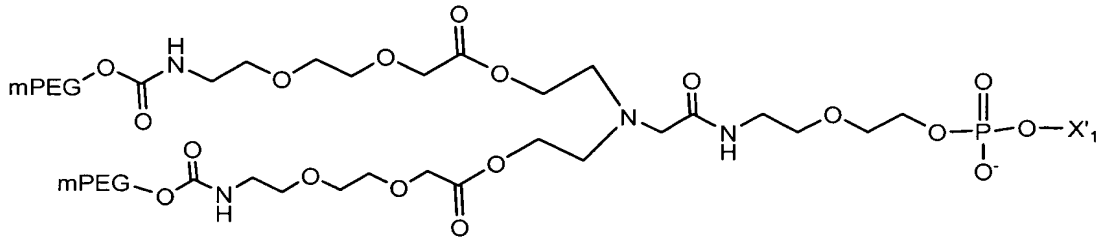

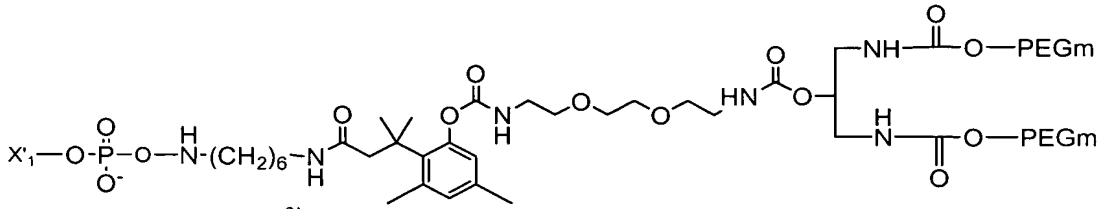

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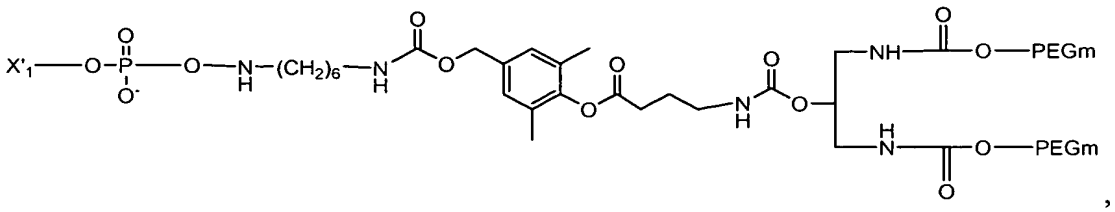

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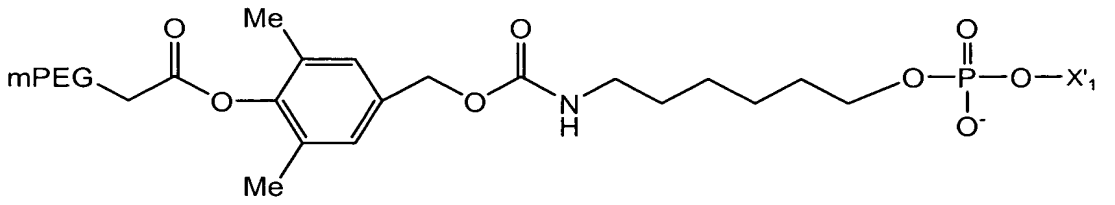

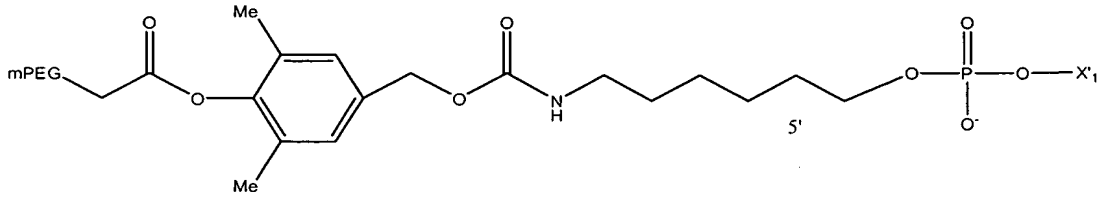

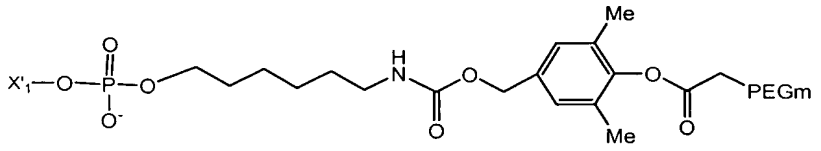

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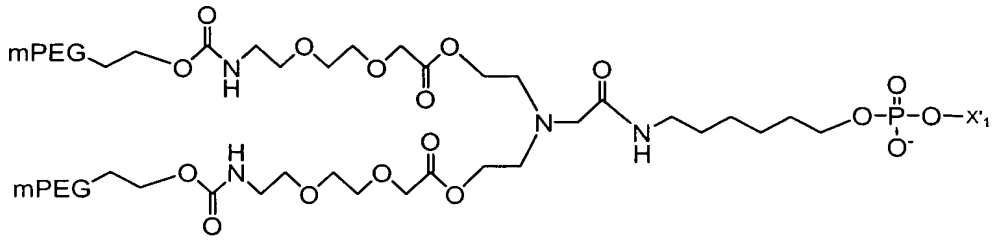

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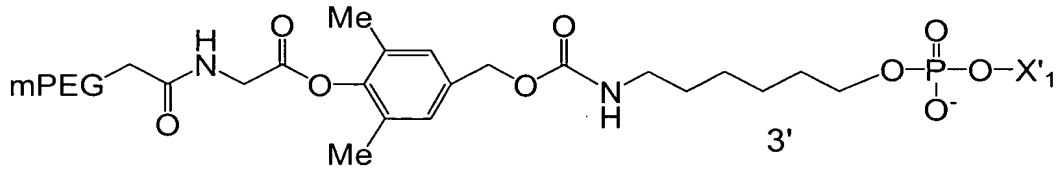

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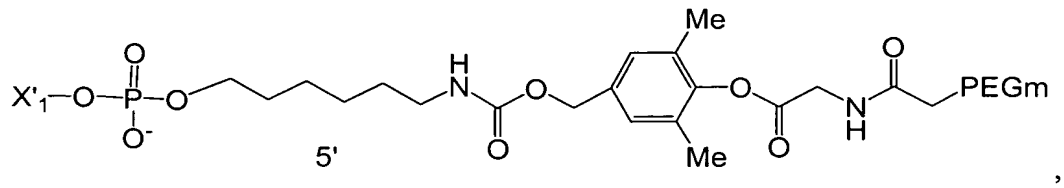

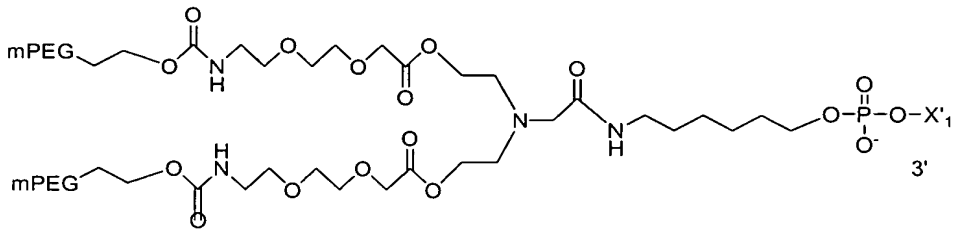

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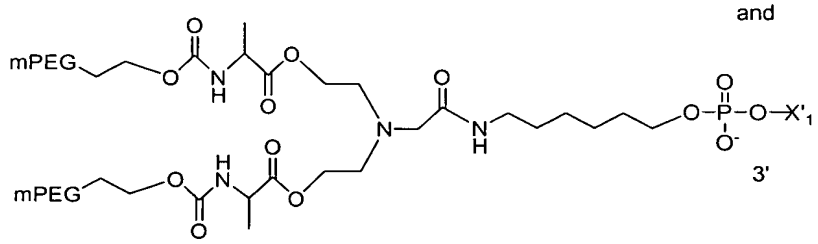
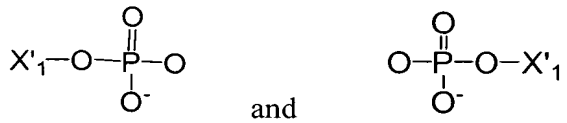


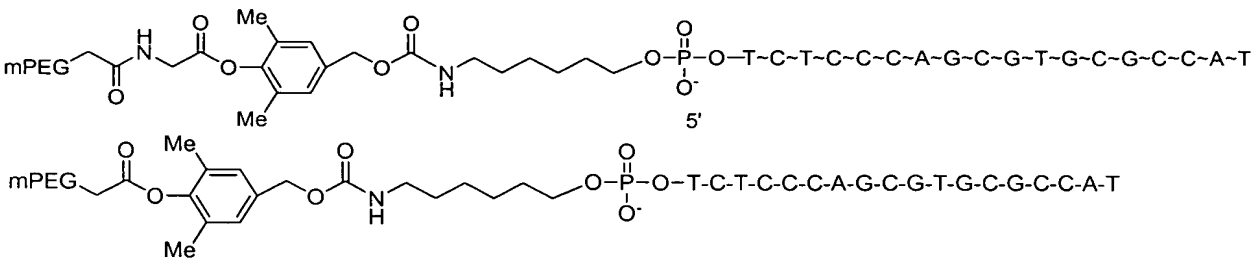
wherein:

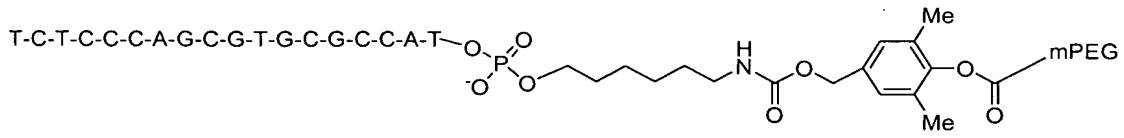


5' 3'

represent an oligonucleotide and point of terminal phosphate modification and mPEG is CH_3-O-(CH_2-CH_2-O)$_X$- ; wherein x is a positive integer selected from about 10 to about 2300.

More preferred compounds of the invention include:





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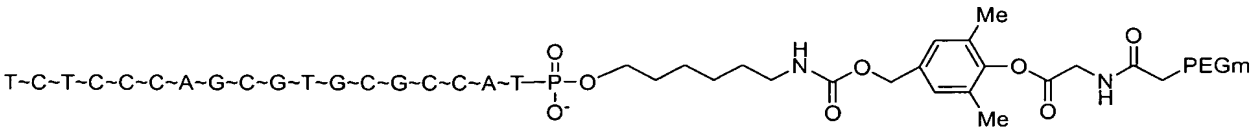


G. METHODS OF TREATMENT

Another aspect of the present invention provides methods of treatment for various medical conditions in mammals. The methods include administering to the mammal in need of such treatment, an effective amount of an oligonucleotide prodrug, which has been prepared as described herein. The compositions are useful for, among other things, treating neoplastic disease, reducing tumor burden, preventing metastasis of neoplasms and preventing recurrences of tumor/neoplastic growths, liver diseases, viral diseases such as, HIV, in mammals. The prodrugs of the present invention can be used for whatever indication the native olignucleotide or antisense oligonucleotides are used for, *i.e.* cancer therapy, etc. Simply by way of example, the inventive prodrugs are contemplated to be employed in the treatment of multiple myeloma, chronic lymphocytic leukemia, non-small cell lung cancer, small cell lung cancer, prostate cancer and other tumors or cancers too numerous to mention.

The amount of the prodrug administered will depend upon the parent molecule included therein an condition being treated. Generally, the amount of prodrug used in the treatment methods is that amount which effectively achieves the desired therapeutic result in mammals. Naturally, the dosages of the various prodrug compounds will vary somewhat depending upon the parent compound, rate of in vivo hydrolysis, molecular weight of the polymer, etc. The range set forth above is illustrative and those skilled in the art will determine the optimal dosing

of the prodrug selected based on clinical experience and the treatment indication. Actual dosages will be apparent to the artisan without undue experimentation.

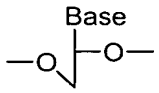
The prodrugs of the present invention can be included in one or more suitable pharmaceutical compositions for administration to mammals. The pharmaceutical compositions may be in the form of a solution, suspension, tablet, capsule or the like, prepared according to methods well known in the art. It is also contemplated that administration of such compositions may be by the oral and/or parenteral routes depending upon the needs of the artisan. A solution and/or suspension of the composition may be utilized, for example, as a carrier vehicle for injection or infiltration of the composition by any art known methods, *e.g.*, by intravenous, intramuscular, subdermal injection and the like.

Such administration may also be by infusion into a body space or cavity, as well as by inhalation and/or intranasal routes. In preferred aspects of the invention, however, the prodrugs are parenterally administered to mammals in need thereof.

It is also contemplated that the prodrugs of the invention be administered in combination (*e.g.*, simultaneously and/or sequentially) with other art-known anticancer agents. Suitable anticancer agents include, simply by way of example: (Paclitaxel; Bristol Myers Squibb); Camptosar® (Irinotecan; Pfizer.); Gleevec® (Imatiinib Mesylate; Novartis); Rituxan® (Rituximab; Genentech/ IDEC); Fludara® (Fludarabine; Berlex Labs); Cytoxan® (cyclophosphamide; Bristol Myers Squibb); Taxotere® (Docetaxel; Aventis Pharmaceuticals); Mylotarg® (Gemtuzumab ozogamicin; Wyeth-Ayerst); Cytosine arabinoside and/or dexamethasone, to name but a few such agents.

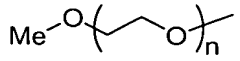
EXAMPLES

The following examples serve to provide further appreciation of the invention but are not meant in any way to restrict the effective scope of the invention. The underlined and bold-faced numbers recited in the Examples correspond to those shown in the Figures. In each of the figures, the sugar moiety and phosphate backbone are represented as:



Base = A, G, C, or T

or simply as —.

The mPEG designation shall be understood to represent 

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General Procedures. All the conjugation reactions between PEG linkers and oligonucleotides were carried out in PBS buffer systems at room temperature. Extraction with organic solvents in general removed the un-reacted oligonucleotides, further anion-exchange chromatography separated PEG-Oligo conjugates from non-reacted excess PEG linkers to give pure products.

HPLC methods. The reaction mixtures and the purity of intermediates and final products were monitored by a Beckman Coulter System Gold® HPLC instrument employing a ZORBAX® 300 SB C-8 reversed phase column (150 x 4.6 mm) or a Phenomenex Jupiter® 300A C18 reversed phase column (150 x 4.6 mm) with a multiwavelength UV detector, using a gradient of 30-90 % of acetonitrile in 0.5 % trifluoroacetic acid (TFA) and 25-35 % acetonitrile in 50 mM TEAA buffer with 4 mM TBACl at a flow rate of 1 mL/min. The anion exchange chromatography was run on Bio-Cad 700E Perfusion Chromatography Workstation from Applied Biosystems using either Poros 50HQ strong anion exchange resin from Applied Biosystems or DEAE Sepharose fast flow weak anion exchange resin from Amersham Biosciences packed in an AP-Empty glass column from Waters. Desalting was achieved by using HiPrep 26/10 or PD-10 desalting columns from Amersham Biosciences.

Example 1

Compound 3. A solution of compound **1** (440 mg, 0.036 mmol) and **2** (5 mg, 3.6 μmol) in PBS buffer (10 mL, pH 7.4) was stirred at room temperature for 12 hrs. The reaction solution was extracted with methylene chloride (DCM, 3 x 10 mL) and the combined organic layers were dried ($MgSO_4$), filtered, and the solvent evaporated under reduced pressure. The residue was dissolved in double distilled water (1.5 mL per 100 mg) and loaded on a HQ/10 Poros strong anion exchange column (10 mm x 60 mm, bed volume ~6 mL). The un-reacted PEG linkers were eluted with water (3 ~ 4 column volumes) and the product then eluted with 0.2 M

NH_4HCO_3 solution (~ 2 column volumes). The fractions containing pure product were pooled and lyophilized to yield pure **3** (19 mg, 1.44 mmol, 40 %).

Examples 2-6

Compounds 5, 7, 9, 11, and **12** were made and purified in the similar way as **3** in the yields ranging from 30 % to 50 %.

Example 7

Compound 14. To a solution of compound **13** (10 mg, 1.7 μmol) in PBS buffer (5 mL, pH 7.4) was added **10** (175 mg, 85 μmol) in five equivalent portions every hour and stirred at room temperature for 12 hrs. The reaction solution was extracted with DCM (3 x 10 mL) and brine (10 mL), and the combined organic layers were dried ($MgSO_4$), filtered, and the solvent evaporated under reduced pressure. The residue was dissolved in double distilled water (1.5 mL) and loaded on a DEAE fast flow, weak anion exchange column (10 mm x 60 mm, bed volume ~ 6 mL) which was pre-equilibrated with 20 mM tris-HCl buffer, pH 7.4. The un-reacted PEG linkers were eluted with water (3 to 4 column volumes) and the product then eluted with a gradient of 0 to100 % 1 M NaCl in 20mM Tris-HCl buffer 7.4 in 10 min, followed by 100% 1M NaCl for 10 min at a flow rate of 3 mL/min. The fractions containing pure product were pooled and desalted on a PD-10 desalting column with 0.2 M NH_4HCO_3 solution (~ 2 column volumes) and the resulting solution was lyophilized to yield pure **14** (25 mg, 0.95 μmol, 57 %).

Example 8

Compound 16 was made and purified the same way as **14**, with a 60% yield.

Example 9

Compound 17. To a solution of AS1 (5 mg, 0.85 μmol) in phosphate buffer (2 mL, pH 7.8) was added **10** (175 mg, 0.085 mmol) which was divided into five equivalent portions in 2 hrs and the resulting solution stirred at room temperature for another 2 hrs. The reaction solution was extracted with DCM (3 x 6 mL) and brine (5 mL), and the combined organic layers were dried ($MgSO_4$), filtered, and the solvent evaporated under reduced pressure. The residue was dissolved in double distilled water (5 mL) and loaded on a DEAE fast flow, weak

anion exchange column (10 mm x 60 mm, bed volume ~ 6 mL) which was pre-equilibrated with 20 mM tris-HCl buffer, pH 7.4. The unreacted PEG linkers were eluted with water (3 to 4 column volumes) and the product then eluted with a gradient of 0 to100% of 1 M NaCl in 20mM Tris-HCl buffer, pH 7.4, for 10 minutes followed by 100% 1M NaCl at a flow rate of 3 mL/min for 10 minutes. The fractions containing pure product were pooled and desalted on a PD-10 desalting column and the resulting solution was lyophilized to yield pure **17** (15 mg, 0.57 μmol, 67 %).

Examples 10-11

Compounds 18 and **19** were made and purified the same way as **17** with yields of 67% for the final products using AS2 and AS3 in place of AS1.

Examples 12-15

Compound 21 was made and purified the same way as **14** by replacing **12** with **20** with a yield of 90%.

Compound 22 was made and purified in the same way as **14** by replacing **12** with **20** with a yield of 65%.

Compound 24 was made and purified in the same way as **14** by replacing **12** with **23** and, for desalting, water (~ 2 column volumes) was used to elute the product instead of 0.2 M NH_4HCO_3 solution. The final was 30%.

Compound 26 was made and purified in the same way as **24** by replacing **23** with **25**. The yield was 30%.

Example 16

Compound 27. To a solution of **13** (10 mg, 1.7 μmol) in phosphate buffer (5 mL, pH 8.5) was added **27** (180 mg, 0.084 mmol) which was divided into ten equivalent portions, and the resulting solution stirred at room temperature for 4 days. The reaction solution was extracted with DCM (3 x 10 mL) and brine (10 mL), and the combined organic layers were dried ($MgSO_4$), filtered, and the solvent evaporated under reduced pressure. The residue was dissolved in double distilled water (1.5 mL) and loaded on a DEAE fast flow, weak anion exchange column (10 mm x 60 mm, bed volume ~ 6 mL) which was pre-equilibrated with 20 mM tris-HCl buffer, pH 7.4. The un-reacted PEG linkers were eluted with water

(3 to 4 column volumes) and the product then eluted with a gradient of 0 to100 % 1 M NaCl in 20mM Tris-HCl buffer, pH 7.4, for 10 min, followed by 100% 1M NaCl at a flow rate of 3 mL/min for 10 minutes. The fractions containing pure product were pooled and desalted on a PD-10 desalting column and the resulting solution was lyophilized to yield **14** (105 mg, 0.0102 mmol, 60 %). The purity of the product was determined by HPLC.

Examples 17-21

Compounds 29, 30 and **31** were made and purified the same way as **28**, except that **13** was replaced by AS1, AS2 and AS3, respectively. A yield of 65% was obtained for each of the final products.

Compound 33 was made and purified the same way as **14** with **32** replacing **12** resulting in a yield of 76%.

Compound 35 was made and purified the same way as **14** except that activated PEG **34** was used in place of **10**. The final yield was 30%.

BIOLOGICAL DATA

Some *in vitro* properties of PEG-Oligo conjugates are summarized below:

Table 4. *In vitro* properties of PEG-Oligo conjugates

Compound	$t_{1/2}$ (buffer)	$t_{1/2}$ (Rat Plasma)	$t_{1/2}$ (PE I)	$t_{1/2}$ (PE II)	$t_{1/2}$ (NucleaseP1)
3	>>24 h	0.7 h	< 5 min	>24 h	< 5 min
7	>> 24 h	0.5 h	< 5 min	>24 h	< 5 min
5	>>24 h	0.5 h	< 5 min	>24 h	< 5 min
9	>> 24 h	3.7 h	< 5 min	36	0.3h
11	>>24 h	2.3 h	< 5 min	63	0.3h
12	>> 24 h	10.6 h	< 5 min	1.7	< 5 min
14	>>24 h	14.7 h	< 5 min	38.9	0.3h
16	>> 24 h	13.8 h	< 5 min	42.7	0.3h
21	>>24 h	6.5h	>24h	>24 h	<5 min
24	>>24 h	11.h	18.7h	36.8h	<5 min
28	>>24 h	>48h	>24h	>48 h	<5 min
33	>> 24 h	>48h	>24h	>48 h	<5 min
35	>> 24 h	4.1h	5.6 h	>48h	<5 min

PEI = Phosphodiesterase I, 5'-exonuclease, kinetics done in pH 8.8 TEAA buffer at 37 °C; PEII = Phosphodiesterase II, 3'-exonuclease, kinetics done in pH 6.5 TEAA buffer at 37 °C; Nuclease P1 = endonuclease, kinetics done in pH 5.3 TEAA buffer at 37 °C; for all enzymes 1 unit releases 1 μmol of oligonucleotide.

Pharmacokinetics of PEG-Oligo Conjugate in ICR Mice

General procedures.

1) Animal Husbandry: Mice were housed 6 per cage, in breeder boxes. Cages were sized in accordance with the "Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resource", National Research Council.

2) Diet: The mice had access to tap water and were fed commercially available lab chow *ad libitum*.

3) Compound Preparation: Compound **13** was dissolved in 4.0 mL of saline and compound **14** was dissolved in 4.1 mL of saline.

4) Administration Site: Compound **13** and **14** were administered as a single dose (Day 1) via the tail vein.

Experimental Design

Sixty (60) mice were assigned, dosed and bled according to the following design, shown by Table 5, below.

Table 5

Grp	Tx	N	Dose (mg/kg)	Dose* (mg/kg)	Inj	Time Points Bled (h)									Vol (μl)
1	**14**	3	120	4	iv	0.03									1000
2	**14**	3	120	4	iv		0.25								1000
3	**14**	3	120	4	iv			0.5							1000
4	**14**	3	120	4	iv				1						1000
5	**14**	3	120	4	iv					3					1000
6	**14**	3	120	4	iv						6				1000
7	**14**	3	120	4	iv							24			1000
8	**14**	3	120	4	iv								48		1000
9	**14**	3	120	4	iv									72	1000
10	**14**	3	120	4	iv									96	1000
11	**13**	3	4	4	iv	0.03									1000
12	**13**	3	4	4	iv		0.25								1000
13	**13**	3	4	4	iv			0.5							1000
14	**13**	3	4	4	iv				1						1000

15	**13**	3	4	4	iv					3						1000
16	**13**	3	4	4	iv						6					1000
17	**13**	3	4	4	iv							24				1000
18	**13**	3	4	4	iv								48			1000
19	**13**	3	4	4	iv									72		1000
20	**13**	3	4	4	iv										96	1000

*oligo equivalent

Three (3) untreated mice were bled via cardiac puncture into EDTA containing tubes for the collection of untreated control plasma.

The mice were injected intravenously with 100 μL per mouse with native compound **13** and **14**. Following sedation with 0.09% Avertin, the mice were terminally bled ~1000 μL by cardiac puncture. Blood was collected into EDTA containing vials. The plasma was collected following centrifugation of the blood and immediately frozen at –80°C on dry ice.

Clinical Examinations:

The mice were examined visually once daily following infusion of the test article, for mortality and signs of reaction to treatment. Any death and clinical signs was recorded. Body weights were measured prior on the day of injection only.

The pharmacokinetic studies for compounds **28** and **33** were done in a similar fashion.

Experimental results

The pharmacokinetic results are summarized in the following Table 6, below.

Table 6. In vivo properties of PEG-Oligo conjugates

Compound	Cmax (μg/mL)	Plasma Half-life (hr)	AUC (hr•μg/mL)
13	14.9	0.19	4.1
14	54.8	0.66	51.8
28	491.2	1.05	730.6
33	556.7	0.25	191.0

Examples 22 - 25

Confirmation of *In Vitro Activity* of Antisense PEG Conjugates

Bcl-2 protein has been shown to have significant anti-apoptotic activity in prostate cancer cells. Downregulation of bcl-2 protein in prostate cancer cells is confirmed by cell death, and induction of cell death by bcl-2 antisense PEG conjugates was employed to confirm the successful intra-cellular delivery of the antisense oligonucleotides.

Materials And Methods For Examples 22 - 25

The tested compounds are listed by Table 6, below.

Table 7

Compound	Description
14	5' G aromatic releasable
28	5'- permanent
33	5' G-->A aromatic releasable
24	24k-mPEG-BCN3-5' aliphatic releasable
35	20k-mPEG-RNL9 5' releasable (intermediate $t_{1/2}$ in rats)

These were prepared as described *supra.*

Cell culture

Mycoplasma-free PC3 cells were obtained from the American Type Culture Collection (Rockville, MD) were grown in Roswell Park Memorial Institute media ("RPMI") (Invitrogen, Grand Island, NY) plus 10% fetal bovine serum ("FBS"), containing 10% (v/v) heat inactivated (56°C) FBS supplemented with 1% non-essential amino acids, 1% pyruvate, 25 mM HEPES (N-2-Hydroxyehtylpiperazine-N'-2-ethanesulfonic acid) buffer, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. Stock cultures were maintained at 37°C in a humidified 5% CO_2 incubator.

Reagents

FBS and Lipofectin (liposome formulation of the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride) were purchased from Invitrogen (Grand Island, NY). The anti-bcl-2 monoclonal antibody was from Dako (Carpinteria, CA). The anti-α-tubulin monoclonal antibody and 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide ("MTT") were purchased from Sigma-Aldrich (St. Louis, MO). Phosphorothioate oligonucleotides were synthesized and purified via standard procedures.

Oligonucleotide transfection

Cells were seeded the day before the experiment in 6-well plates at a density of 25 x 104 cells per well, to be 60-70% confluent on the day of the experiment. All transfections were performed in Opti-MEM medium in the absence of FBS and antibiotics as per the manufacturer's instructions. The appropriate quantities of reagents were diluted in 100 µl of Opti-MEM medium to give a final concentration of Lipofectin and oligonucleotide. The solutions were mixed gently and preincubated at room temperature for 30 minutes to allow complexes to form. Then, 800 µl of Opti-MEM was added, the solution mixed, and overlaid on the cells that had been pre-washed with Opti-MEM. The incubation time for oligonucleotide/ Lipofectin complexes in Opti-MEM was 24 hrs, followed by incubation in complete media containing 10% FBS. The total incubation time before cell lysis and protein isolation was usually 72 h at 37°C.

Western Blot Analysis

Cells treated with oligonucleotide-lipid complexes were washed in PBS and then extracted in lysis buffer [50 mM Tris-HC1 pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaC1, 1 mM EGTA, 50 µg/ml Pefabloc SC, 15 µg/ml aprotinin, leupeptin, chymostatin, pepstatin A, 1 mM Na3VO4, 1 mM NaF] at 4°C for 1 h. Cell debris was removed by centrifugation at 14 000 g for 20 rain at 4°C. Protein concentrations were determined using the Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, CA). Aliquots of cell extracts, containing 25-40 µg of protein, were resolved by SDS-PAGE, and then transferred to Hybond ECL filter paper (Amersham, Arlington Heights, IL), and the filters incubated at room temperature for 1-2 hr in 5% BSA in PBS containing 0.5% Tween-20. The filters were then probed with 1:500 dilutions of the anti bcl-2 antibody in 5% BSA in PBS containing 0.5% Tween-20 at 4°C overnight. After washing in PBS containing 0.5% Tween-20, the filters were incubated for 1 hour at room

temperature in 5% milk in PBS containing 0.5% Tween with a 1:3,000 dilution of a peroxidase-conjugated secondary antibody (Amersham). After washing (3 x 10 min), electrochem-iluminescense ("ECL") was performed according to the manufacturer's instructions.

Determination of Rate of Cell Proliferation

The effect on cellular viability of PEG conjugates was determined by MTT assay. Briefly, 15-20 x 10^4 cells were seeded in 6-well plates and allowed to attach overnight. Cells were then treated with the appropriate concentrations of oligonucleotide complexed to Lipofectin for 24 hrs at 37°C, followed by incubation in complete media (100 µl) containing 10% FBS. Cell viability was determined daily. Ten µl of 5 mg/ml MTT in PBS was added to each well, followed by incubation for 4 h at 37°C. Then, 100 µl of 10% SDS in 0.04 M HCl was added to each well, followed by incubation for overnight at 37°C to dissolve the formazan crystals. Absorbance was determined at 570 mn with a Benchmark plus Microplate spectrophotometer (Bio Rad, Hercules, CA). Experiments were performed in 6 replicates, and data are presented as the average +/- S.D.

Quantitation of intracellular ROS levels

2',7'-dichlorodihydrofiuorescein diacetate (H_2DCF-DA) and dihydroethidium (HE) were used to determined reactive oxygen species ("ROS") and superoxide levels. Both dyes are nonfluorescent and can freely diffuse into cells. When HE is oxidized to ethidium (E), it intercalates into cellular DNA and fluoresces. Oxidation of H_2DCF-DA yields 2'-7' dichlorofluorescein (DCF), which also fluoresces, and both can be detected by flow cytometry. Cells were harvested by trypsinization, washed with PBS and stained with 50 µM H_2DCF-DA or 50 µM HE in phenol red-free DMEM for 2 h at 37°C. The mean fluorescence channel numbers of DCF and E were analyzed by flow cytometry in the FL-1 and FL-2 channels respectively. A minimum of 10,000 cells were acquired for each sample and data were analyzed using CELLQuest software (Becton Dickinson). Histograms were plotted on a logarithmic scale.

Example 22

Inhibition Of Bcl-2 Protein Expression

Three PEG oligonucleotides (compounds **14**, **28** and **33**) targeted to bcl-2 expression were transfected into PC3 cells and their capacity to inhibit bcl-2 protein expression was evaluated by Western blotting.

Effects of Lipofectin

Initially, the degree of inhibition of bcl-2 protein expression in PC3 cells induced by compound **14** was determined in the presence and absence of Lipofectin. PC3 cells were treated with compound **14** (at 200, 400 and 800 nM) in the presence or absence of Lipofectin for 24 hrs in Opti-MEM, and then for a further 67 hrs in complete media. Protein samples (30-40 μg of protein/lane) were analyzed by Western blotting as described in the Material and Methods, with tubulin used as a control protein species. The % inhibition vs. control, untreated cells was determined by laser-scanning densitometry. The Western Blot results are shown by FIG. 12.

The expression of α-tubulin and PKC-α was unchanged, confirming that Lipofectin is helpful to obtaining penetration of compound 14 into PC-3 cells, and confirming that only bcl-2 protein expression was downregulated by compound 14.

Further investigation demonstrated that compounds **14** and **28** were the most active at 400 nM. PC3 cells were treated with complexes of compound **14**, compound **28**, and compound 23 at 400, 800 and I000 nM and Lipofectin for 24 hrs in Opti-MEM, and then for a further 67 hrs in complete media. Protein samples (30-40 μg of protein/lane) were analyzed by Western blotting as described in the Material and Methods, with tubulin used as a control protein species. The % inhibition vs. control, untreated cells was determined by laser-scanning densitometry.

Compound **14** produced 86% downregulation and compound **28** produced 78% downregulation.

Example 23

Dose-dependent analysis of bcl-2 protein expression by PEG oligonucleotides

To further confirm the inhibitory effect of compounds **14** and **28** on bcl-2 protein expression, PC3 cells were treated with increasing concentrations (25, 50, 100, 200 and 400 nM) of compound **14**, compound **28**, and compound **13** as a positive control, complexed to Lipofectin, for 24 hrs in Opti-MEM, and then for a further 67 hrs in complete media. Protein samples (30-40 μg of protein/lane) were analyzed by Western blotting as described in the Material and Methods section, *supra*.

A concentration-dependent inhibition of bcl-2 protein expression was observed by Western Blot for compounds 14 and 28, relative to controls. About 1-2% inhibition was observed at 50 nM, increasing to 99% and 75 % at a concentration of 400 nM. For compound **24**, essentially no inhibition was observed at 50 nM, but the inhibition increased to 77% at a concentration of 400 nM. Transfection with compound **13** was used as a positive control. The expression of α-tubulin was not inhibited by any oligonucleotide.

The above experiment was repeated with compound **24** as a control. PC3 cells were treated with increased concentrations of compounds **35** and **24** (25, 50, 100, 200 and 400 nM) were complexed to Lipofectin for 24 hrs in Opti-MEM, and then for a further 67 hrs in complete media. Protein samples (30-40 μg of protein/lane) were analyzed by Western blotting as described in the Material and Methods section, *supra*, with α-tubulin used as a control protein species. The % inhibition vs. control, untreated cells was determined by laser-scanning densitometry.

Example 24

Effect of PEG oligonucleotides on PC3 cell growth

The effects of compounds **14** and compound **28** on the growth of PC3 prostate cancer cells, *in vitro*, was also tested. PC3 cells were treated with oligonucleotide/Lipofectin complexes. As shown in Fig. 10, transfection of

antisense oligonucleotide compound **14** at 400 and 200 nM strongly inhibited cell growth, while compound **28** only slightly affected the proliferation rate.

Example 25

Effect of PEG oligonucleotides on production of reactive oxygen species in PC3 cells

Production of reactive oxygen species or ROS in PC3 cells was evaluated flow cytometrically by two methods. The first was based on oxidation of hydroethidium (HE) to ethidium (E), which subsequently intercalates into DNA with fluorescence detectable by flow cytometry. The second method employed the oxidation of cell-permeable 2',7'-dihydrodichlorofluorescein diacetate to fluorescent 2',7'-dichlorofluorescein (DCF). In PC3 cells, treatment with compound 14/Lipofectin (400 nM/15 μg/ml) complexes for 24 hours in Opti-MEM generated ROS three days later as evaluated by flow cytometrically by both E (1.9-fold increase vs. control, untreated cells) and DCF (2-fold increase vs. control, untreated cells) fluorescence. As confirmed by the data summarized by Fig. 11, compound **28** did not produced any increase in ROS production vs. control, untreated cells. Furthermore, the production of ROS is very closely linked to the rate of cellular proliferation; cells cease growing after treatment with 400 nM of compound **14**, and this oligonucleotide also causes an increase in the production of ROS (DCF and HE).